Intratumoral Genetic Heterogeneity in Sporadic Colorectal Cancer and Its Association with Underlying Genomic Instability.

Izabela Bozena Georgiades

Thesis submitted for the Degree of Doctor of Medicine

University of Edinburgh
2000

TABLE OF CONTENTS

CHAPTER 2.
Materials and methods.

CHAPTER 3.
Spatial and temporal analysis of microsatellite instability in sporadic colorectal cancer.

CHAPTER 4.
Spatial and temporal analysis of chromosomal instability in sporadic colorectal cancer.

DECLARATION

I declare that the work submitted is my own and that the appropriate credit has been given where reference has been made to the work of others.

Izabela B. Georgiades

October, 2000

ABSTRACT

Two major mechanisms of genomic instability, microsatellite instability (MIN), also referred to as a replication error positive phenotype (RER+) and chromosomal instability (CIN), have been identified to date in sporadic colorectal cancer. Each leads to the development of a distinct phenotype of colorectal cancer.

This thesis has analysed genetic intratumoral heterogeneity in 22 sporadic colorectal cancers and its association with two known types of underlying genomic instability. It has also established the relevance of colorectal cancer xenografts as models for investigating the genetics of colorectal cancer.

This study has identified the presence of genetic intratumoral heterogeneity in sporadic colorectal cancer. It has shown that the RER+ phenotype is characteristic of a proportion of sporadic colorectal cancer and confirmed that a single sample analysis is sufficient for determining RER+ phenotype in these tumours. The analysis of chromosome copy number changes in the RER- and RER+ groups of sporadic colorectal cancers confirmed previously reported higher incidence of chromosomal abnormalities occurring in RER- cancers. Different patterns of chromosomal changes were found to occur in RER- and RER+ tumours. In this study the most frequently detected chromosomal abnormalities in RER- and RER+ cancers were 20q+, 18q-, 13q+, 8p-, 1p-, 8q+ and 1p-, 19del respectively.

The most important finding of this study is the identification of a novel group of sporadic colorectal cancers which do not display instability of either chromosomes or microsatellites (called non-MIN, non-CIN cancers). These tumours do not show any striking differences in clinical and pathological features compared with RER- tumours exhibiting high levels of chromosomal instability, but may harbour fewer abnormalities of p53. It is likely that non-MIN, non-CIN colorectal cancers represent a distinct entity in sporadic colorectal cancer and, based on this data, their prevalence might be as high as 35% of RER- colorectal cancers or 25% of sporadic colorectal cancers in total.

The analysis of colorectal cancer xenografts established from samples collected from multiple sites from primary tumours showed that a xenograft established from a

single sample is in general representative of its tumour of origin, despite the presence of genetic heterogeneity within primary tumours. This applies firstly to the preservation of the RER+ and RER- phenotype and secondly to specific chromosomal abnormalities being retained in RER- colorectal cancer xenografts. The study also showed that p53 status of the primary tumour is unchanged in a corresponding xenograft and that the DNA ploidy closely resembles that of the sample of origin. Preservation of all these important genetic features in colorectal cancer xenografts makes them a valuable model for investigating the genetics of the disease.

The results of this study provided new valuable information on genetic intratumoral heterogeneity in sporadic colorectal cancer and its association with different mechanisms of underlying genetic instability. Although this work has not addressed directly the issue of response to therapy, the data provided will be important in establishing whether classification of genetic instability in this way has a bearing on response to different therapeutic agents.

ACKNOWLEDGEMENTS

First of all I would like to thank Professor Andrew Wyllie who was responsible for initiation of this project, who has provided guidance and support throughout and supplied invaluable help with the laborious process of writing up. My advisor Professor David Harrison for his support and Professor Colin Bird for useful comments on the manuscript. I am especially grateful to Lucy Curtis for assistance with CGH and valuable advice throughout this project and Robert Morris for guidance in many technical aspects of the work. I would like to thank Joan Flannigan for help with sample collection and Jennifer Doig for assistance with the xenograft handling. Thanks are due to Sula Corbet for constructive comments on data analysis and the manuscript and Scott Bader and Wael abdel-Rahman for extremely helpful and inspiring discussions. I would like to acknowledge the contribution of Mr Peter Han from the Police Forensic Science Laboratory in DNA fingerprinting of samples and the staff of the research laboratory in the Department of Pathology at the Western Infirmary in Glasgow for immunohistochemical detection of the Ebstein-Barr virus in one of the samples.

I am very grateful to Steve Evans for proof-reading of the manuscript of this thesis.

I would like to thank Dr James and Bozena Bain and the Cancer Research Campaign for providing funding for this work.

My late husband John provided a continuous encouragement and support for my work for which I am extremely grateful.

DEDICATION

to

John

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1.

Introduction.

1.1. Epidemiology and clinical aspects of colorectal cancer.

1.1.1. Incidence, mortality and survival rates.

Large bowel cancer is the second most common cancer in Britain. Each year 31,200 new cases are diagnosed in the UK. It constitutes 12% of all malignant neoplasms, excluding non-melanoma skin cancer (NMSK) in females where it is preceded only by breast cancer. In males it closely follows lung and prostate cancer accounting for 13% of all malignancies, excluding NMSK.

Peak incidence for colorectal carcinoma occurs in the age group 60 to 79 years, and fewer than 20% of cases occur before the age of 50 years.

Cancer is the cause of a quarter of all deaths in the UK, 11% of which are due to large bowel cancer. Five-year age standardised relative survival rates in adults diagnosed with colorectal cancer during 1986-90 in England and Wales are 39% for females and 38% for males (Cancer Research Campaign Scientific Yearbook, 1999).

1.1.2. Distribution.

Colorectal carcinoma death rates vary considerably world-wide with the highest rates in the United States and Eastern Europe and up to tenfold lower rates in Mexico, South America and Africa. Environmental factors, particularly diet, are implicated as major factors contributing to these striking geographic differences (Crawford, 1999).

Within Britain colorectal cancer is more common in Scotland than it is in England or Wales (Cancer Research Campaign Scientific Yearbook, 1999).

1.1.3. Etiologic factors.

1.1.3.1. Diet and colorectal cancer.

The evidence supporting the role of diet as an important etiologic factor in the genesis of colorectal cancer comes from observations of different incidence rates in different populations. Comparisons of migrant groups show lower rates of colon cancer in Chinese native to Asia compared with Chinese born in America; studies of populations with defined dietary patterns, i.e. vegetarians, show rates lower than those in the general population (Motwani *et al.*, 1997).

Although the data gathered from a large number of case-control and cohort studies over the past decade are not entirely consistent, several important risk factors have emerged. The dietary factors most frequently implicated as predisposing to a higher incidence of colorectal cancer are excess energy intake relative to requirements, a low content of unabsorbable vegetable fibre, a high content of highly refined carbohydrates in the diet, intake of red meat and decreased intake of protective micronutrients (see Giovannucci and Willett, 1994 for review).

Dietary components affect the biochemical composition of faecal content but direct mechanisms by which they exert their effect on the large bowel mucosa are poorly understood. Some substances present in the diet are highly mutagenic. These include fecapentaenes, that can also be derived from the action of *Bacteroides* species on faecal content, and heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), present in fried or heavily browned meats. Increased dietary fat may cause increased secretion of bile acids which have the potential to act as tumour promoters and co-carcinogens. The direct effect of fibre is to reduce colon transit time, thus both the effective contact time between putative carcinogens in the faecal content and the colonic mucosa and the time for gut microflora to act on faecal content to generate additional possible carcinogens such as fecapentaenes is reduced. Fibre also reduces faecal bile acid levels and colonic pH, a higher level of which is postulated to contribute to carcinogenic activity (Motwani *et al.*, 1997).

Dietary factors also appear to have a different impact in different parts of the large bowel. Cancers of proximal colon (caecum, ascending and transverse colon up to the

splenic flexure) are associated with intrinsic factors such as bile acids and sex hormones. Cancers located in the distal part of the large bowel (descending and sigmoid colon and rectum) are more closely related to diet and cigarette smoking (reviewed by Breivik and Gaudernack, 1999). Alcohol intake may enhance risk of cancer in the distal colorectum and its influence is particularly strong when combined with a diet low in methionine and folate (see Giovannucci and Willett, 1994 for review).

1.1.3.2. Precancerous lesions and conditions preceding colorectal carcinoma.

The precancerous nature of colorectal epithelial polyps has been debated for years and still remains controversial. Although circumstantial, there is evidence that most colorectal carcinomas arise from pre-existing adenomatous polyps. Here some well-documented relevant facts are presented (reviewed by Rosai, 1996).

1. Solitary hyperplastic polyps, retention polyps and the polyps of Peutz-Jeghers do not become malignant or, if they do, the rate is negligible.
2. Patients with any type of polyposis syndrome are at increased risk for the development of large bowel carcinoma.
3. Villous adenomas become malignant in a high proportion of cases (29% to 70%).
4. Adenomatous polyps can undergo malignant transformation as documented by adenomatous polyps with focal carcinoma.
5. Not all adenomatous polyps become malignant within the normal life span of an individual.
6. There is an overwhelming parallelism between adenomatous polyps and colonic carcinoma. Populations that have a high incidence of polyps also have a high incidence of carcinomas and vice versa. Large bowels with carcinomas have a higher incidence of polyps elsewhere in the specimen than those without carcinoma. Adenomatous polyps are a good epidemiologic indicator of colon carcinoma risk. The morphologic, histochemical (mucin stains), immunochemical (CEA, blood group substances), flow cytometric, nuclear morphometric, and ultrastructural features of adenocarcinomas, and the most atypical areas of adenomatous polyps are extremely similar.

7. The malignant transformation of adenomatous polyps (so-called adenoma-carcinoma sequence) have been documented with chemically induced colorectal tumours in animals and has become a paradigm of the process of malignant transformation of epithelial tissue. It has been demonstrated that the morphologic progression from adenomatous polyp with mild to moderate to severe atypia and to invasive and metastatic carcinoma is accompanied (and presumably caused) by a series of molecular alterations.
8. Colonoscopic polypectomy results in a lower-than-expected incidence of colorectal carcinoma in the population subjected to this procedure.
9. There is evidence suggesting that some colorectal carcinomas arise de novo rather than on the basis of pre-existing polyps.

Apart from adenomatous lesions there are a number of pathological conditions associated with elevated risk of developing colorectal carcinoma. Although sporadic colorectal carcinoma accounts for the vast majority of all colorectal cancer cases diagnosed, in a proportion of cases familial syndromes substantially increase the risk of encountering the disease. Familial adenomatous polyposis coli (FAP) almost inevitably leads to development of colorectal cancer. FAP and hereditary non-polyposis colorectal cancer (HNPCC) represent two highly penetrant autosomal dominant predisposition syndromes responsible for 2-10% of colorectal cancer cases. The risk in patients with juvenile polyposis is at least as high as 10%. Peutz-Jeghers syndrome presents only a slight risk and although occasionally malignant change has been described in metaplastic ploys, the risk of colorectal cancer occurrence has not been shown to be higher than for normal colorectal mucosa. Both types of inflammatory bowel disease, ulcerative colitis and Crohn's disease, predispose to colorectal cancer. Surgical procedures altering the colonic microenvironment (urethrosigmoidostomy, cholecystectomy and gastrectomy) as well as anastomoses are associated with an increased incidence of colorectal cancer.

The majority of precancerous lesions and conditions show various degrees of the common histological change - dysplasia, and the risk magnitude of malignant transformation appears to be closely related to the grade of dysplasia.

1.1.4. Clinical aspects of colorectal carcinoma.

1.1.4.1. Clinical features.

Two-thirds of the large bowel cancers are located in the rectum, rectosigmoid or sigmoid colon with the one-third distributed in the remainder of the colon.

Symptoms develop late and usually accompany the advanced stages of the disease. Ceacal and right colonic cancers often grow as bulky readily bleeding lesions and are called to clinical attention by the appearance of fatigue, weakness and iron deficiency anaemia. Left-sided tumours produce occult bleeding, changes in bowel habits or left-lower-quadrant discomfort. Cancers of rectum and sigmoid colon, despite causing prominent disturbances in bowel function, tend to be more infiltrative at the time of diagnosis than proximal lesions and have somewhat poorer prognosis. Patients who present with iron deficiency anaemia of unknown origin should be always carefully examined, preferably by colonoscopy, to exclude colorectal carcinoma as a cause (Crawford, 1999).

1.1.4.2. Staging.

Various colorectal cancer classification systems are in use, but all take into account the depth of invasion into the bowel wall, spread into the adjacent organs, involvement of regional lymph nodes and the presence of distant metastases. For the purpose of this study the widely used Dukes' system was employed.

Dukes' classification:

Dukes A : Tumour confined to the bowel wall, may involve the muscularis propria but not beyond.

Dukes B : Tumour spreads to extramural tissue, involves the serosa or mesenteric fat tissue, but there is no lymph node involvement.

Dukes C : Any degree of bowel wall involvement with regional lymph node metastasis or tumour extends beyond contiguous tissue or immediately adjacent organs with no regional lymph node involvement.

Dukes D : Any invasion of bowel wall with or without regional lymph node metastasis with evidence of distant metastasis.

1.1.4.3. Morphology.

Most cancers in the large bowel are adenocarcinomas. The microscopic characteristics of right- and left-sided colorectal carcinomas are similar. Differentiation may range from well differentiated tumours closely resembling the adenomatous lesions but now invading the submucosa to undifferentiated, anaplastic tumours. Approximately 20% of colorectal adenocarcinomas are well differentiated, 60% moderately differentiated and 20% poorly differentiated. Between 10-15% of colorectal cancers are mucinous. They show two main growth patterns: (1) glands filled with mucin together with interstitial mucin and (2) chains or clumps of cells surrounded by mucin. Although many colorectal tumours produce mucin only those carcinomas in which extracellular mucin constitutes more than 60% of the cancer volume in adequate numbers of sections are formally classified as mucinous. Mucinous cancers have in general worse prognosis. Secretion of mucin is thought to facilitate the extension of the malignancy by dissecting the bowel wall (Crawford, 1999). However, recent studies suggest that mucinous histotype is not an independent prognostic factor and that the shorter survival rate observed in mucinous colorectal cancers is attributable to a more advanced stage of presentation (Messerini *et al.*, 1999). In fact, mucinous carcinomas more frequently show microsatellite instability (MIN) than non-mucinous cancers (Messerini *et al.*, 1997) and MIN tumours show a tendency towards increased patient survival (Thibodeau *et al.*, 1993; Lothe *et al.*, 1993; Bubb *et al.*, 1996).

Colorectal tumours spread by direct extension through the submucosa, muscularis propria and serosa into adjacent structures. The metastases occur through lymphatic channels and blood vessels in the regional lymph nodes, liver, lungs and bones followed by many other sites. In general the disease has spread beyond the range of curative surgery at the time of diagnosis in 25-30% of patients (Crawford, 1999).

1.1.4.4. Treatment and prognosis.

Early stages of the disease are treated primarily by surgery. If at the time of diagnosis regional or distant metastases are discovered, a combination of surgical and other therapeutic modalities is indicated. Adjuvant therapies include pre- and postoperative radiotherapy, chemotherapy and immunotherapy. At present, most patients with Dukes A colorectal cancer do not have sufficiently high statistical risk of recurrence to justify postsurgical adjuvant treatment to the entire group. A significant proportion of these patients however will ultimately develop regional recurrence or metastatic disease. It would be highly beneficial to identify additional prognostic markers, such as specific genetic defects facilitating invasion or metastases, to enable the application of adjuvant intervention in a selective fashion.

The estimated survival at 5 years is in excess of 85% among patients with large bowel cancer diagnosed in localised state (Dukes' A). Survival ranges from 20-80% in the group of patients diagnosed with regional involvement (Dukes' B and C) and is directly proportional to the number of involved lymph nodes, serosal involvement, tumour grade and patient's age. The 5-year survival is 10% or less among patients diagnosed with distant disease (Dukes' D) (Motwani *et al.*, 1997).

1.2. The genetic basis of colorectal cancer.

1.2.1. Introduction.

Despite variations in the gross and microscopic appearances of colorectal cancers they share certain characteristics. They have several phenotypic attributes of a malignant neoplasm such as excessive growth, local invasiveness, and the ability to form distant metastases. These phenotypic characteristics are acquired in a stepwise fashion - a phenomenon called tumour progression, and result from accumulation of specific genetic lesions.

Neoplastic transformation is increasingly being interpreted in terms of evolutionary mechanisms and it is generally agreed that cancer development is a multistep process involving natural selection of mutations occurring at the somatic level. A number of reports reveal distinct patterns of genetic alterations in tumours of different histogenesis (Mertens *et al.*, 1997, Shackney and Shankey, 1997) indicating that specific genetic alterations are not random but may be closely related to different types of tissue of origin and exposure to specific carcinogens.

Here, the most common genetic changes encountered in colorectal cancers as well as mechanisms implicated in colorectal carcinogenesis are reviewed.

1.2.2. Genetic changes acquired during the adenoma-carcinoma sequence.

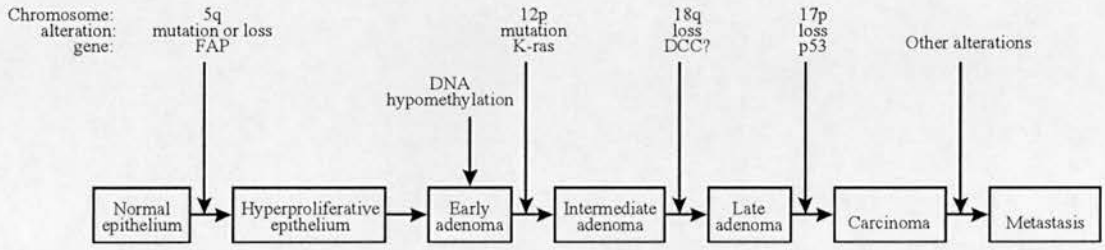
Colorectal carcinomas represent human tumours probably most intensely investigated at the genetic level. The fact that most of them arise from pre-existing, benign lesions - adenomas, and gradually progress through increase in size and acquisition of dysplastic features to malignant lesions, permits the investigation of genetic changes at different stages of tumour development and progression. The adenoma-carcinoma sequence provides an excellent system in which the nature and order of acquisition of specific genetic alterations can be studied.

Although tumourigenesis has long been thought to be a multistep process, the first genetic model of colorectal carcinogenesis was presented by Fearon and Vogelstein in 1990 (Fearon and Vogelstein, 1990). It was proposed that:

1. Colorectal carcinomas arise as a result of mutational activation of oncogenes coupled with mutational inactivation of tumour suppressor genes.
2. Most colorectal carcinomas probably arise from a minimum of five or more genetic alterations, while adenomas appear to require correspondingly fewer alterations.
3. Total accumulation of changes rather then their order is responsible for determining tumour's biologic properties.
4. Some mutant tumour suppressor genes appear to exert a phenotypic effect in a heterozygous state indicating their dominant properties at the cellular level.

The following genetic model for colorectal carcinogenesis was proposed (Figure 1).

Figure 1. *A genetic model for colorectal carcinogenesis.*



This model was subsequently modified, as new tumour suppressor genes, oncogenes and genes involved in controlling apoptosis were showed to play an important role in colorectal carcinogenesis. The major breakthrough in understanding the mechanisms responsible for acquisition of numerous genetic alterations required for the development of a malignant neoplasm came with a discovery that defects in "caretaker" genes responsible for maintenance of genome integrity, substantially facilitate carcinogenesis. The notion that the spontaneous mutation rate in somatic cells is not sufficient for multiple mutations observed in many human malignancies and that a mutator phenotype might be required for multistep tumorigenesis has been argued for years (Nowell, 1976, Loeb, 1991; Hartwell, 1992). However, only the

studies of the involvement of mismatch repair (MMR) genes in HNPCC and a subset of sporadic colorectal cancers provided evidence that mutation rates in tumour cells with MMR deficiency are substantially higher then in normal cells (Bhattacharyya *et al.*, 1994; Shibata *et al.*, 1994; Eshleman *et al.*, 1995). Two distinct phenotypes were subsequently described in sporadic colorectal cancer. The first, affecting a small fraction of tumours, known as microsatellite instability (MIN) or replication error positive phenotype (RER+). This manifests itself as a high rate of alteration in the length of short tandemly repeated nucleotide sequences (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Eshleman *et al.*, 1995) and is consequent upon defects in DNA mismatch repair system. The second, believed to affect the majority of tumours, replication error negative phenotype (RER-), shows major abnormalities in chromosome structure and number and has been suggested to arise through chromosomal instability (CIN) (Langauer *et al.*, 1997b). These two distinct colorectal cancer phenotypes are suggested to arise through two distinct pathways and have been subsequently shown to target different subsets of oncogenes and tumour suppressor genes (Olschwang *et al.*, 1997; Salahshor *et al.*, 1999).

1.2.3. Tumour suppressor genes in colorectal carcinogenesis.

1.2.3.1. The role of APC gene in familial and sporadic colorectal cancer.

Familial adenomatous polyposis coli is an autosomal dominantly inherited disease that affects 1 in 7000 individuals. Patients with FAP develop hundreds to thousands of large bowel adenomas during their second and third decade of life. Their large number virtually guarantees that some will progress to invasive carcinomas. FAP patients often also develop extracolonic manifestations such as retinal lesions (congenital hypertrophy of the retinal pigment epithelium - CHRPE), osteomas, desmoids of the skin, and brain tumours.

The search for the gene responsible for FAP was directed toward the chromosomal region on the short arm of chromosome 5, where cytogenetically evident deletion was observed (Herrera *et al.*, 1986). Further molecular studies demonstrated tight linkage of the disease to markers on 5q21 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). The

adenomatous polyposis coli (*APC*) gene was finally identified in 1991 using positional cloning (Groden *et al.*, 1991; Kinzler *et al.*, 1991; Nishisho *et al.*, 1991). Patients with FAP carry a germline mutation in one copy of *APC*. Inactivation of the second copy is required for initiation of tumour development. Complete *APC* inactivation has been found in the earliest neoplastic lesions called dysplastic aberrant crypt foci (ACF), which are believed to be precursors of adenomas (Smith *et al.*, 1994), but the exact mechanism of tumour initiation by *APC* mutation is as yet unclear.

APC is speculated to act as a "gatekeeper" of colonic epithelial proliferation (Kinzler and Vogelstein, 1997) inactivation of which is required for net cellular proliferation. This hypothesis is supported by the fact that the APC protein is located at the basolateral membrane in colorectal epithelial cells with expression more pronounced as cells migrate up through the crypt column (Smith *et al.*, 1993; Miyashiro *et al.*, 1995). Expression of wild-type APC in colorectal epithelial cells with *APC* mutations results in apoptosis, suggesting that *APC* may control cell death process (Morin *et al.*, 1996). The *APC* gene encodes a 310kDa cytoplasmic protein consisting of 2843 aminoacids, with many structural and binding domains, however without strong similarities to proteins of known function. APC binds with high affinity to β- and γ-, but not α-catenin, members of a family of proteins associated with intercellular adhesion (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). The catenins are part of desmosomal and adherens junction complexes and bind to the cytoplasmic domain of E-cadherin - a calcium dependent cell-cell adhesion molecule (see Cowin, 1994 for review). Although APC is not found in a cadherin complex its association with components of this structure suggests that its role in the generation of neoplasia may occur through the modulation of cell-cell interactions. APC has also been shown to interact with EB-1 a highly conserved 30kDa protein of unknown function (Su *et al.*, 1995). It has been suggested that mutant APC protein may interfere with the function of wild-type protein through the formation of partially insoluble aggregates and therefore function not only as a tumour suppressor gene but act in the dominant negative fashion described for p53 (Howe and Guillem, 1997). Mutations of *APC* cause aberrant accumulation of β-catenin which then binds T cell factor-4 (Tcf-4)

causing increased transcriptional activation of unknown genes. *c-MYC* oncogene has been identified as one of the target genes in this signalling pathway (He *et al.*, 1998).

The role of *APC* in the development of colorectal adenomas and carcinomas is supported by a knockout mouse model. The *m*ultiple *i*nterstinal *n*eoplasia mouse (*Min*) is characterised by a dominantly inherited phenotype caused by a nonsense mutation at codon 850 in one copy of the murine *APC* gene (Su *et al.*, 1992). *Min* mice develop multiple bowel polyps in a similar manner to FAP patients. In contrast to the human disease, these are found not only in the colon but also throughout the small interstine. The severity with which the mice are affected is modulated by an unlinked locus known as *m*odifier *o*f *m*in 1 (*Mom1*). This gene is homologous to the human gene encoding type II non-pancreatic phospholipase A2 (*PLA2s*). It has been suggested that the action of *PLA2s* is complex (see also 1.2.3.4) and affects lipid homeostasis and digestion of dietary fats as well as maintenance of normal bacterial flora and elimination of aberrant crypt cells (MacPhee *et al.*, 1995).

The study of *APC* gene in sporadic colorectal cancer revealed the presence of somatic mutation in the great majority of tumours (Miyoshi *et al.*, 1992; Powell *et al.*, 1992). Previous studies frequently demonstrated deletions in the 5q21 region in sporadic colorectal carcinomas (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988). Mutations in the *APC* gene occur with the same frequency in carcinomas and in nonmalignant adenomas as small as 5 mm suggesting that loss of *APC* function is one of the earliest steps in sporadic colonic neoplasia (Powell *et al.*, 1992). There is conflicting data published on the frequency of mutations in the *APC* gene in sporadic RER+ and RER- colorectal cancers. It has been found to appear equally frequently in RER+ and RER- tumours in some studies (Huang *et al.*, 1996), while other investigators have shown the *APC* mutations to be more frequent in RER- (Olschwang *et al.*, 1997) or RER+ (Konishi *et al.*, 1996) sporadic colorectal cancers. Due to these discrepancies it is difficult to speculate on the relative importance of inactivation of this particular tumour suppressor gene in two different pathways of colorectal carcinogenesis.

1.2.3.2. The p53 gene.

Mutations in the *p53* gene are the most common genetic aberrations currently detected in various types of human malignant tumours (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Levine *et al.*, 1991 and 1994). The Li-Fraumeni syndrome is an autosomal dominantly inherited syndrome caused by a germ-line mutation in the *p53* gene. Patients with this syndrome develop numerous carcinomas and sarcomas as well as leukemias, but rarely colorectal carcinomas. The *p53* gene is located on the short arm of chromosome 17 (Isobe *et al.*, 1986) and encodes a nuclear phosphoprotein which has ability to bind to DNA in a sequence-specific manner and acts as a DNA-dependent transcription factor. It is involved in a wide range of cellular functions, most important of which is the maintenance of genomic stability. For this reason *p53* has been named "guardian of the genome".

Evidence from mice null for the *p53* gene, which develop normally but have a high incidence of tumour development in adulthood (Donehower *et al.*, 1992), suggests the function of *p53* in the protection of cells after genomic damage. Indeed, certain types of DNA damage induce *p53* and lead to cell cycle arrest allowing for repair of DNA damage preventing the passage of potentially harmful genetic mutations to daughter cells. Alternatively, p53 can induce apoptosis in these cells with the same end result (Fritsche *et al.*, 1993). The mechanisms of p53-induced cell cycle arrest and apoptosis are complex and involve numerous components. The *p53* mediated G_1 arrest requires transcriptional induction of $p21^{WAF1/CIP1}$ gene (el-Deiry *et al.*, 1994), while *p53*-dependent apoptosis appears to be related to modulation of the function of the members of the *Bcl-2* gene family, transcriptional activation of *Bax* (apoptosis promoter) in particular (Miyashita and Reed, 1995). It is generally accepted that *p53* plays a critical role in maintaining genomic integrity (Kastan *et al.*, 1991; Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Nelson and Kastan, 1994). Association of *p53* defects with chromosomal instability is well documented (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993; Deangelis *et al.*, 1993; Meling *et al.*, 1993; Carder *et al.*, 1995; Bouffler *et al.*, 1995; Donehower *et al.*, 1995; Gualberto *et al.*, 1998; Venkatachalam *et al.*, 1998). However, RER+ colorectal cancer cell lines with *p53* mutations have been shown to remain

chromosomally stable (Eshleman *et al.*, 1998a) indicating that *p53* mutation does not invariably induce chromosomal instability. There are also implications of p53 involvement in the regulation of *hMSH2* and it therefore has a possible role in mismatch repair (Schrerer *et al.*, 1996).

Loss of *p53* function occurs frequently in sporadic colorectal cancer and is found in up to 75% of cases (Baker *et al.*, 1990; Cunningham *et al.*, 1992; Vandenbroek *et al.*, 1993; Khine *et al.*, 1994). It has also been shown to be a rather late event in colorectal carcinogenesis, occurring usually at the transition from the late adenoma to carcinoma (Vogelstein *et al.*, 1988; Baker *et al.*, 1990; Fearon and Vogelstein, 1990). *p53* mutations detected in colorectal carcinomas are mostly missense mutations. They often result in the nuclear accumulation of abnormal protein with increased half-life, allowing detection by immunohistochemical methods. It has also been shown that 90% of all mutations in the *p53* gene are located in exons 5-8 (Levine *et al.*, 1991). Although, according to Knudson's two-hit hypothesis (Knudson, 1971), defects in both copies of a tumour suppressor gene are required for its inactivation, some mutations in the *p53* gene appear to exert a dominant negative effect (Eliyahu *et al.*, 1988; Milner and Medcalf, 1991). An oncogenic form of *p53*, with a missense mutation which confers a dominant gain-of-function phenotype disrupting spindle checkpoint control leading to genomic instability has also been described (Gualberto *et al.*, 1998).

Although published reports of the overall frequency of *p53* mutations in sporadic colorectal cancer are similar, conflicting data has appeared with regard to the frequency of *p53* mutations in RER- and RER+ colorectal carcinomas (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Kim *et al.*, 1994; Ilyas *et al.*, 1996; Cottu *et al.*, 1996; Remvikos *et al.*, 1997). Recent reports indicate that *p53* mutations are significantly less common in RER+ than in RER- colorectal cancers (Cottu *et al.*, 1996; Eshleman *et al.*, 1998a) suggesting that its inactivation is not necessary for tumour progression in colorectal cancers with microsatellite instability.

1.2.3.3. DCC: the Deleted in Colon Cancer gene.

DCC was one of the earliest genes found to be associated with colorectal cancer. Deletions of chromosome 18q, to which *DCC* was subsequently mapped in the region 18q21, are very common in colorectal carcinomas (Vogelstein *et al.*, 1988).

DCC is a large gene that extends over 1.35 million bases and has at least 29 exons (Fearon *et al.*, 1990). It encodes a cell adhesion protein predicted to have 1447 amino-acids which resembles most a neural cell adhesion molecule. *DCC* is normally widely expressed on the colon mucosa (Hedric *et al.*, 1994) and has been demonstrated to mediate epithelial-epithelial and epithelial-mesenchymal interactions (Chuong *et al.*, 1994). It was hypothesised that loss of *DCC* might result in loss of cell-to-cell contact, thereby enhancing metastasis. Indeed *DCC* expression is reduced or absent in 70-75% of colon cancers (Vogelstein *et al.*, 1988; Fearon *et al.*, 1990; Itoh *et al.*, 1993; Cho *et al.*, 1994). Loss of heterozygosity in 18q21 region is seen in 11% of adenomas and in 47% of adenomas with microinvasion, suggesting that it is a late alteration that might be involved in the progression of colorectal cancer. Complete or partial loss of 18q is also significantly more frequent in advanced stages of the disease (Jen *et al.*, 1994) and 18q deletions are indicative of poor prognosis (Kern *et al.*, 1989). Recently described mutant mice lacking both alleles of *DCC* however show no abnormalities of intestinal biology (Fazeli *et al.*, 1997), questioning the role of *DCC* in colorectal carcinogenesis.

Studies of the frequency of inactivation of the *DCC* gene in RER+ and RER- colorectal cancers have shown the loss or reduction of *DCC* mRNA expression and allelic loss at the *DCC* locus to be significantly less frequent in RER+ cancers compared to RER- tumours, suggesting its limited role in the tumourigenesis of colorectal cancers with microsatellite instability (Yamamoto *et al.*, 1998a).

1.2.3.4. TGF-β R II: Transforming Growth Factor β type II receptor (RII).

TGF-β is a potent inhibitor of epithelial cell growth (Roberts *et al.*, 1985) and can induce apoptosis of normal colonic epithelial cells (Wang *et al.*, 1995). Loss of responsiveness to TGF-β is common in human cancers and is thought to be an

important step in tumourigenesis. Most colon cancer cells are resistant to the anti-proliferative effects of TGF-β (Hoosein *et al.*, 1989).

The intracellular biological effects of TGF-β are initiated following ligand binding to oligomeric complexes of high affinity TGF-β type I and type II receptors. If one of the receptors is absent or inactivated, the cells lose their responsiveness to TGF-β. The mammalian type II receptor for TGF-β is a transmembrane serine/threonine kinase (Mathew and Vale, 1991; Lin *et al.*, 1992). Signalling by this receptor is mediated by Smad protein family which upon phosphorylation by activated receptors form complexes move to the nucleus associate with DNA-binding proteins and activate gene transcription (Massague *et al.*, 1997).

Inactivation of the type II TGF-β receptor has been reported in colon cancer cells with microsatellite instability (Markowitz *et al.*, 1995; Parsons *et al.*, 1995; Akiyama *et al.*, 1996; Togo *et al.*, 1996a). Mutations of *TGF-β* receptor II are frequently found in adenomas and are present in the vast majority of carcinomas from HNPCC patients where they appear to be an early event in cancer progression (Lu *et al.*, 1996; Akiyama *et al.*, 1997). *RII* mutations in colon cancers with microsatellite instability usually result in frameshifts clustered in a naturally occurring 10-bp microsatellite-like polyadenine tract at codons 125-128 of its 565-codon open reading frame (*BAT-RII*). In a few MIN colon cancers inactivation of one of the *RII* alleles occurs via non-*BAT-RII* mutations that alter the RII kinase domain (Markowitz *et al.*, 1995; Parsons *et al.*, 1995) demonstrating an underlying selective advantage for RII inactivation. This is irrespective of whether it occurs via *BAT-RII* or non-*BAT-RII* mutational events. In most MIN colorectal cancers the polyadenine tract mutations affect both alleles of *RII* suggesting tumour suppressor activity of wild-type RII (Parsons *et al.*, 1995). Recently inactivation of TGF-β receptor II through non-*BAT-RII* point mutations has been found to occur in 15% of RER- colorectal carcinomas. In addition another 55% of RER- cancers demonstrate a transforming growth factor β signalling blockade distal to RII (Grady *et al.*, 1999) indicating that TGF-β pathway is a major target for inactivation in both RER+ and RER- colorectal cancers.

1.2.3.5. Other putative tumour suppressor genes in colorectal cancer.

*M*utated in *C*olorectal *C*ancer gene (*MCC*) was first identified as a candidate gene responsible for FAP due to its location in the 5q21 region (Kinzler *et al.*, 1991). The protein encoded by *MCC* shows sequence homology to a murine G protein receptor, important in signal transduction. However, no germline mutations were detected in *MCC* in FAP patients eliminating the possibility that defects in *MCC* cause FAP syndrome (Nishisho *et al.*, 1991). Although loss of heterozygosity (LOH) in the *MCC* locus is frequently detected in colorectal adenocarcinomas, independent loss of *MCC* without *APC* is a rare event and in cases where allele loss occurs mutation of the retained allele is uncommon (Curtis *et al.*, 1994). This suggests that *MCC* does not function as an independent tumour suppressor in the majority of sporadic colorectal cancers.

BAX is a 21 kDa protein, a member of the BCL2 family of proteins which controls an important checkpoint prior to activation of the caspase family of proteases in apoptosis. Unlike BAX-BCL2 heterodimers which appear to promote cell survival, BAX-BAX homodimers are potent death inducers (Webb *et al.*, 1997) and a significant effector in the initiation of apoptosis. Inactivating mutations in *BAX* have been observed in a proportion of colorectal cancers (Rampino *et al.*, 1997; Yagi *et al.*, 1998; Yamamoto *et al.*, 1998; Ouyang *et al.*, 1998). These mutations are restricted to tumours with mismatch repair deficiency and usually occur in G_8 tract of exon 3 (Rampino *et al.*, 1997). It has been argued that selective loss of a death pathway in these tumours, due to *BAX* inactivation, represents a critical event in early carcinogenesis. However, the G_8 tract is a classical target site for nucleotide mismatch and it has been shown that *BAX* mutation, in a proportion of RER+ colorectal cancers, is not necessary for formation of the founder clone and can occur later in the tumour progression (Abdel-Rahman *et al.*, 1999).

The tumour suppressor gene *p16* located on the short arm of chromosome 9 (9p21) encodes for a cyclin-dependent kinase 4 (CDK4) inhibitor that binds to and inactivates cyclin D/CDK4 complex responsible for phosphorylation of the retinoblastoma gene

product. Hypophosphorylated retinoblastoma protein blocks the transcription of important cell-cycle regulatory proteins and is critical in inhibition of cellular proliferation. In human neoplasms *p16* is silenced in three ways; by homozygous deletion, methylation of the promoter, and point mutation. The first two mechanisms comprise the majority of inactivation events in most tumours. Additionally, the loss of *p16* may be an early event in cancer progression, because deletion of at least one copy is high in some premalignant lesions (see Liggett and Sidransky, 1998 for review). Inactivation of *p16* through hypermethylation of its promoter region has been found in most sporadic colorectal cancers with microsatellite instability and in a small proportion of RER- tumours (Toyota *et al.*, 1999). However, the exact frequency of *p16* inactivation through all three mechanisms has not been established in colorectal cancer.

18q21 region has been shown to contain another gene that was found to be frequently deleted in pancreatic cancer and therefore named *D*eleted in *P*ancreatic *C*ancer locus 4 (*DPC4*). Subsequently, 16% of colon cancers were demonstrated to have mutations in the *DPC4* gene (Takagi *et al.*, 1996). The significance of *DPC4* lies in its homology to *Drosophila melanogaster* gene *MAD* (*M*others *a*gainst *d*pp). Mutations in *MAD* and *dpp* lead to defects in midgut morphogenesis and dorsal-ventral pattering during embryogenesis. The dpp protein is a member of a family related to TGF-β, a potent inhibitor of epithelial cells growth. *MAD* appears to function downstream of *dpp* in the signalling pathway. At least two other components related to *MAD* called *MADR2* (Eppert *et al.*, 1996) and *SMAD4* (Takagi *et al.*, 1996; Thiagalingam *et al.*, 1996) have been identified and both have been mapped to 18q21. *MADR2* and *SMAD4* are known to be central players in the signal transduction pathway activated in response to the large family of TGFβ-like ligands.

Partial deletion in the short arm of chromosome 1 occurs in up to half of all colorectal cancers (Leister *et al.*, 1990; Muleris *et al.*, 1990; Couturier-Turpin *et al.*, 1992; Bardi *et al.*, 1993 and 1995; Bomme *et al.*, 1996; di Vinci *et al.*, 1996) and has been shown to be an early event in colorectal carcinogenesis (di Vinci *et al.*, 1998). It is

also an indicator of poor prognosis (Ogunbiyi *et al.*, 1997). One of the three commonly deleted regions in chromosome 1 overlaps with the region to which *PLA2s* has been mapped. *PLA2s* encodes type II non-pancreatic phospholipase A2 (MacPhee *et al.*, 1995) one of the enzymes responsible for the production of arachidonic acid (a prostaglandin precursor). It is not clear however how loss of its function contributes to tumour development (see also 1.2.3.1). 1p36 region contains another candidate for a tumour suppressor gene - *p73*. This gene encodes a protein highly homologous to p53 (Kaghad *et al.*, 1997) but subsequent search for somatic mutations in *p73* gene in colorectal carcinomas revealed that such mutations are extremely rare (Han *et al.*, 1999). Human aflatoxin B-1 aldehyde reductase gene located at 1p35-1p36 has also been suggested to play a role in colorectal carcinogenesis due to its presumptive involvement in detoxification of genotoxic and cytotoxic substances (Praml *et al.*, 1998). Other putative tumour suppressor genes located on the short arm of chromosome 1 include *ID3* (inhibitor of DNA binding 3; 1p36.13 - p36.12), *NB/NBS* (neuroblastoma suppressor; 1p36.13 - p36.11), *TNFR2* (tumour necrosis factor receptor 2; 1p36.3 - p36.2), *DAN* (differential-screening-selected gene aberrant in neuroblastoma; 1p36.13 - p36.11), *CDC2L1* (cell division cycle 2-like 1; 1p36) and *BRCD2* (breast cancer suppressor-2; 1p36) (Knuutila *et al.*, 1999) but their role in colorectal carcinogenesis is unclear.

Allelic loss on chromosome 8p is found in about half of all sporadic colorectal carcinomas (Cunningham *et al.*, 1993) however no tumour suppressor genes have been convincingly identified in this region. Possible candidates include *FEZ1* gene encoding Fez1 protein containing leucine-zipper region with similarity to the DNA-binding domain of the cAMP-responsive activating-transcription factor 5. *FEZ1* gene transcripts are undetectable in more than 60% of epithelial tumours and mutations in *FEZ1* have been found in oesophageal cancers and prostate cancer cell lines, suggesting that its inactivation may play a role in development of various human tumours (Ishii *et al.*, 1999). Other possible candidates include a gene frequently deleted in human liver cancer *DLC1* (*d*ynein *l*ight-*c*hain gene *1*;

8p21.3-p22), *PRLTS* (*P*DGF-*r*eceptor β-*l*ike *t*umour *s*uppressor), *EXT1* and *EXTL3* [exostoses (multiple)-like 3] (Knuutila *et al.*, 1999).

1.2.4. Oncogenes in colorectal carcinogenesis.

1.2.4.1. The ras oncogene and receptor-mediated signal transduction pathways.

Ras is a member of a large family of genes highly conserved between species encoding for guanosine triphosphate (GTP) binding proteins involved in many processes including transmission of extracellular signals. The p21ras protein is the product of three homologous genes, *H-ras*, *N-ras* and *K-ras*. It is located on the inner surface of the plasma membrane where it takes part in the transduction of growth and differentiation signals. Although the *ras* genes are highly homologous only the *K-ras* gene, located in the short arm of chromosome 12 (12p12), plays a significant role in the development of colorectal cancer with approximately 50% of the tumours carrying mutations in one of the gene alleles (Vogelstein *et al.*, 1988). *K-ras* mutations are almost always localised to one of the three codons, with codon 12 being most frequently affected. Few mutations are found in codons 13 and 61. Mutations in the *K-ras* gene cause the protein to be constitutively active in the GTP-bound state, leading to a continually growth-stimulated state. Alteration in the *K-ras* gene were found with similar frequency in large adenomas (>1cm) and carcinomas in cancer bearing bowels, 42% and 47% respectively, but in only 10% of small adenomas obtained mostly from FAP patients (Vogelstein *et al.*, 1988), placing this genetic event relatively early in colorectal carcinogenesis, between an early and a late adenoma. However, there is evidence suggesting that *K-ras* mutations might occur even earlier since they were detected in 57% to 73% of aberrant crypt foci (Pretlow *et al.*, 1993; Losi *et al.*, 1996).

Activation of the *K-ras* oncogene appears to be equally important in the development of RER+ and RER- colorectal cancers (Olschwang *et al.*, 1997). Although other reports indicate that mutations in the *K-ras* gene are significantly more frequent in RER- cancers (Konishi *et al.*, 1996).

1.2.4.2. c-myc.

c-myc encodes a 62 kDa phosphoprotein localised to both the cytoplasm and nucleus which as a heterodimer with a nuclear protein Max binds to DNA and can activate transcription. Activation of *c-myc* with increased levels of both mRNA and protein has been associated with cellular proliferation in numerous tissues. Rearrangements and amplifications of the *c-myc* gene are responsible for elevated expression of the c-myc protein. In normal colonic mucosa c-myc protein is present in the proliferative compartment in the lower one third of the crypts. Elevated levels of c-myc protein have been described in colorectal carcinomas (Melhem *et al.*, 1992; Royds *et al.*, 1992). Recently previously enigmatic overexpression of *c-myc* in colorectal carcinomas have been elucidated by the discovery of its interactions with APC and β-catenin through Tcf-4 signalling pathway. Expression of *c-myc* gene was shown to be repressed by wild-type APC and activated by β-catenin which aberrant accumulation is caused by mutations of *APC*. These effects were mediated through Tcf-4 binding sites in the *c-myc* promoter (He *et al.*, 1998).

1.2.4.3. bcl-2.

bcl-2 was originally cloned from a 14;18 translocation breakpoint associated with several human B cell lymphomas (Tsujimoto *et al.*, 1985; Cleary and Smith, 1986). The bcl-2 protein is a member of a large family of homo- and heterodimerising proteins, some of which are survival-supporting, like bcl-2 itself and bcl-xL, whilst others are death promoting (e.g. bax, bad and bcl-xS) (see Webb *et al.*, 1997 for review). Its oncogenic potential has been attributed to its ability to inhibit apoptosis. Expression of *bcl-2* is normally confined to the base of colonic crypts, but with the onset of dysplasia and progression to early adenoma its expression increases and is seen throughout the crypts (Watson *et al.*, 1996). It has been postulated that deranged expression of the bcl-2 protein may cause resistance to apoptosis in colonic epithelial cells and thus confer a survival advantage thereby contributing to local cell proliferation and dysplasia.

1.2.4.4. CD44.

CD44 is a family of type I transmembrane glykoproteins that are widely expressed on a variety of cells including cells of epithelial, mesenchymal, and hematopoietic origin. All members of CD44 family are encoded by a single gene located on the short arm of chromosome 11 (11p13) that consists of 19 exons (Stamenkovic *et al.*, 1989; Screaton *et al.*, 1992). CD44 has been implicated in lymphocyte homing and activation, hematopoiesis, and tumour progression and metastasis. It is believed to function as a cell adhesion receptor linking the cell and the cytoskeleton to extracellular matrix molecules. In the normal colorectal mucosa, CD44 protein is expressed at low levels and is confined to the base of the crypts. In colorectal tumours expression of CD44 protein is generally strongly enhanced in comparison to normal mucosa, although there is marked inter- and intratumoral heterogeneity. The major upregulation of CD44 occurs at the transition from normal mucosa to adenoma, but CD44 overexpression is already present in aberrant crypt foci with dysplasia. This indicates that deregulation of CD44 represents an early event in colorectal carcinogenesis. CD44 upregulation is thought to be direct or indirect result of constitutive activation of the Wnt pathway. Enhanced CD44 expression is seen in tumours from FAP patients with a germline *APC* mutation as well as in *Apc* mutant mice (see Wielenga *et al.*, 2000 for review).

1.2.5. DNA methylation.

The term DNA methylation refers to the methylation of cytosine residues (5-methylocytosisne) at CpG sites which is characteristically clustered to so-called CpG islands in gene promoter regions. Hypo- and hypermethylation of these regions are related to activation and inhibition of transcription and this type of regulation is essential to cell differentiation and embryological development (see Monk, 1995 for review). DNA methylation is closely related to the mechanism by which one copy of a gene is preferentially silenced according to parental origin, generally referred to as genomic imprinting (Feil and Kelsey, 1997). Patterns of DNA methylation are maintained during cell division by 5-cytosine DNA methyltransferase (DNMT). DNMT preferentially methylates hemimethylated CpG sites copying established

methylation patterns to the newly synthesised DNA strands. This ensures that information related to gene expression is maintained through cell division.

Both global hypomethylation compared with normal tissue as well as regional hypermethylation have been observed in human malignancies. Alterations in DNA methylation have been proposed as a central pheneomenon underlying the neoplastic process (Laird and Jaenisch, 1994; Counts and Goodman, 1995; Baylin *et al*, 1998; Breivik and Gaudernack, 1999).

Widespread genomic hypomethylation occurs early in colorectal carcinogenesis with similar levels of hypomethylation found in benign and malignant colorectal neoplasms (Goelz *et al.*, 1985b; Feinberg, 1988). Hypomethylation of proto-oncogenes has been observed in liver tumours and leukemias. A variety of proto-oncogenes such as *c-fos* gene, the *c-myc* gene and the *Ha-ras* and *Ki-ras* genes have shown reduced levels of DNA methylation (see Laird and Jaenisch, 1994 for review). DNA hypomethylation leads to elevated mutation rates in murine embryonic stem cells nullizygous for the major DNA methyltransferase (*Dnmt1*). Gene deletions were found to be predominant mutations in these cells and their major cause was either mitotic recombination or chromosomal loss accompanied by duplication of the remaining chromosome. Both aneuploidy and DNA hypomethylation are observed early in the transformation process. It has therefore been postulated that genomic hypomethylation provides the incipient cancer cells with a mutator phenotype promoting loss of heterozygosity in regions containing tumour suppressor genes (Chen *et al.*, 1998).

There have been many reports of regional increases in DNA methylation levels. Regional hot spots of hypermethylation have been found on chromosome 3p, 11p and 17p in a variety of human tumours. There is evidence for inactivation of tumour-suppressor gene function through hypermethylation of the *Rb* gene in sporadic retinoblastoma. It is possible, therefore, that hypermethylation of the promoter region of tumour suppressor genes leading to gene inactivation results in a selective growth advantage of neoplastic cells (see Laird and Jaenisch, 1994 for review). Tumour suppressor gene methylation in cancer is usually associated with lack of gene transcription and absence of coding region mutation. Thus it has been proposed that methylation of CpG islands serves as an alternative mechanism of gene inactivation in

carcinogenesis (Toyota *et al.*, 1999). It has also been shown that methylation of cytosine to 5mC leads to a higher rate of C→T mutations compared with that of unmethylated sites (Rideout *et al.*, 1990). Recently, it became apparent that hypermethylation of the promoter region of *hMLH1* is responsible for its inactivation and subsequent development of RER+ phenotype in the vast majority of sporadic colorectal cancer with microsatellite instability (Cunningham *et al.*, 1998; Herman *et al.*, 1998; Deng *et al.*, 1999; Maekawa *et al.*, 1999; Wheeler *et al.*, 1999). Two distinct types of hypermethylation in cancer have been described; A - for ageing-specific and C - for cancer specific. Both may have distinct causes and different roles in cancer development. The mechanism of type A methylation is unknown, but it is likely that it results from physiological rather than genetic alteration. Type A methylation is very frequent and present in large numbers of cells, it is present in all individuals, not just patients with cancer; and it is gene and tissue specific. In contrast type C methylation is relatively infrequent in primary colorectal cancer and is never observed in normal colon mucosa. Detailed analysis of type C methylation in colorectal cancers revealed a striking pattern suggesting the presence of hypermethylator phenotype in a subset of sporadic colorectal cancers (Toyota *et al.*, 1999). Through its ability to silence multiple genes simultaneously CpG island methylator phenotype would be functionally equivalent to genetic instability, resulting in rapid accumulation of molecular alterations with a potential to accelerate the neoplastic process.

1.2.6. Genomic instability in colorectal carcinogenesis.

1.2.6.1. Introduction.

Carcinogenesis is a multistep process which is increasingly viewed in terms of evolutionary mechanisms and natural selection at the somatic level. Although, the genes of multicellular organisms exist in an environment that promotes cellular cooperation, the genes in the somatic cells are not exceptions to the general rule of natural selection. Somatic mutations are selected solely based on their ability to improve the reproductive capacity of a somatic clone and natural selection will favour

cells that escape the organism's control mechanisms even if it contradicts the goal of reproduction of the organism.

Evolution through natural selection depends on two essential elements, selection pressure and availability of genetic variation. A selection pressure that favours escape from the organism's growth control mechanisms is an inevitable consequence of multicellularity. A somatic mutation will have a larger potential to reproduce if the cell can escape from the suppressive signals brought on by the other genes and cells of the organism. However, since preservation of genetic homogeneity is crucial for survival of a multicellular organism, a number of homeostatic processes ensuring integrity of the genome have evolved. These mechanisms include antioxidation and detoxofication preventing DNA damage, DNA repair mechanisms, elimination of cells that have sustained unrepairable DNA damage and mechanisms ensuring fidelity of DNA replication. A DNA damaging environment will therefore, exert growth-inhibiting effect on normal cells, and consequently selection pressure that favours escape from these control mechanisms (see Breivik and Gaudernack, 1999 for review).

No evolution can occur without the availability of genetic variation. It has been postulated that spontaneous mutation rate in somatic cells is not sufficient to account for multiple mutations observed in human tumours. An underlying genomic instability (mutator phenotype) is required for the generation of the multiple mutations that underlie cancer (Loeb, 1991). It has been argued that an early step in tumour progression is one that induces a mutator phenotype providing the required genetic variation for natural selection at the somatic level.

This hypothesis has subsequently been proved and two major levels of genomic instability have been identified in human malignances. The first involves subtle sequence changes that alter one or a few DNA base pairs. These mutations usually result from defects in one of the two major DNA repair systems - nucleotide-excision repair and mismatch repair. The second involves changes at chromosomal level including chromosomal gains and losses, chromosome translocations and gene amplifications, the molecular bases of which are not yet clear (see Lengauer *et al.*, 1998 for review). Two kinds of genomic instability have been so far identified in colorectal cancer: microsatellite and chromosomal instability.

Here, the mechanisms implicated in destabilisation of the genome in colorectal cancers are reviewed.

1.2.6.2. The role of defects in mismatch repair system and microsatellite instability in colorectal carcinogenesis.

1.2.6.2.1. Introduction.

Long before the discovery of its involvement in human carcinogenesis, mismatch repair was extensively studied in bacteria. Later it was described in yeast and finally in higher eukaryotes. The information gained from studies of the MMR system in bacteria suddenly become relevant to human neoplasia in 1993 when the RER phenotype of microsatellite instability was discovered in human cancers and was rapidly shown to be due to defects in mismatch repair.

Evidence supporting the role of MMR defects in carcinogenesis includes: (i) theoretical considerations of the requirement for aquisition of a mutator phenotype as an early event in the multistep carcinogenesis, (ii) discovery that MMR defects cause a mutator phenotype destabilising the genome and altering expression of genes integral for carcinogenesis, (iii) presence of defects in MMR genes in the germline of HNPCC kindred members, (iv) finding that such defects behave as classic tumour suppressor genes in both familial and sporadic colorectal cancers, (v) the fact that MMR knockout mice have an increased incidence of tumours, and (vi) that genetic complementation of MMR defective cells stabilises the MMR deficiency-associated microsatellite instability (see Eshleman and Markowitz, 1996 for review).

1.2.6.2.2. MMR system in bacteria, Saccharomyces cerevisiae and its human homologues.

One role of MMR is to recognise and repair mistakes made by DNA polymerases during replication. While the MMR system in bacteria requires 10 independent components there are three critical ones, named after their corresponding bacterial mutator strains: *MutS*, *MutL* and *MutH*. MutS recognises and binds to the mispair or loop, then recruits MutL and MutH to form a complex which scans the duplex for the

nearest hemimethylated site which MutH then nicks on the unmethylated strand. An exonuclease then excises the nascent strand from the nick back past the mismatch. This patch is then resynthesised and ligated.

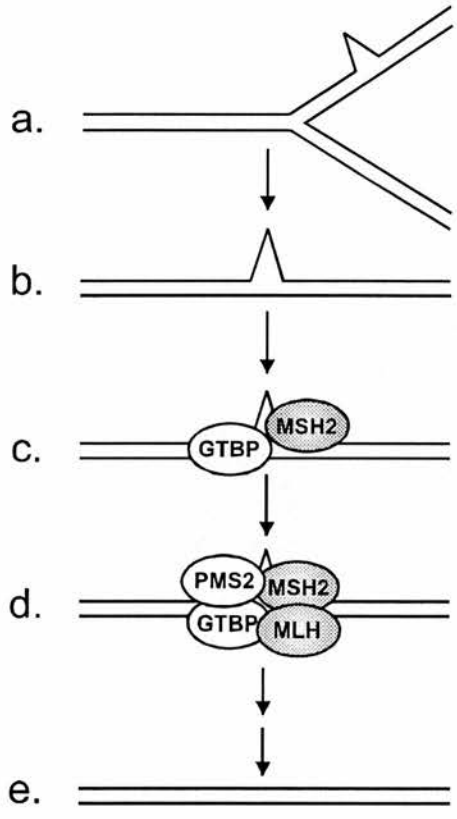
There are extensive similarities between bacterial and human MMR system. Most importantly, both provide the genome with a 100-1000-fold level of protection against mutations arising during DNA replication. A major difference is that the human system has multiple homologues for each bacterial component (Table 1). Human homologues of *MutS* include *hMSH2* (*h*uman *MutS* homologue 2), *GTBP* (G·T mismatch binding protein; also referred to as *hMSH6*) and *hMSH3*. The gene product of *hMSH2* binds the mismatch as heteroduplex with the products of *hMSH6* or *hMSH3*. However, hMSH2 is capable of binding mismatched nucleotides independently and its absence results in an extreme mutator phenotype with numerous mutations in repeat units and increased rate of point mutations (Bhattacharyya *et al*, 1994; Shibata *et al.*, 1994; Boyer *et al.*, 1995). Neither *hMSH6* nor *hMSH3* deficiency is sufficient for establishing strong mutator phenotype indicating a high degree of redundancy in their function (Kolodner, 1995; Risinger *et al.*, 1996; Umar *et al.*, 1998).

Table 1. *Human MMR genes.*

Bacterial MMR homologue	Human gene	Chromosomal localisation	Germline mutations in humans
MutS	*hMSH2*	2p16	yes
	hMSH6 (GTBP)	2p16	yes
	hMSH3	-	-
MutL	*hMLH1*	3p21	yes
	hPMS1	2q31-33	yes
	hPMS2	7p22	yes

Human homologues of *MutL* include *hMLH1* (human *MutL* homologue 1), *hPMS1* (human post-meiotic segregation 1) and *hPMS2*. In *Saccharomyces cerevisiae* (*S. cerevisiae*) MLH1-PMS1 heterodimer interacts with MSH2 bound to a mispaired base which is consistent with the idea that the MLH1-PMS1 complex plays the same role in mismatch repair in eukaryotes as MutL plays in bacteria (see Kolodner, 1995 for review). Figure 2 illustrates MMR pathway in human cells.

Figure 2. *MMR pathway in human cells* (Kinzler *et al.*, 1996).



a,b - During DNA replication single base or larger mismatches can occur due to misincorporation by polymerase or strand slippage.

c - The mismatch is recognised by mutS homologues. In humans optimal mismatch recognition is thought to require at least two *mutS* homologues, hMSH2 and GTBP. hMSH3 may substitute for GTBP in certain cases.

d,e - MutL homologues are then recruited to the complex (d) and the mismatch is repaired (e) by a process that in bacteria involves an exonuclease, helicase II DNA polymerase III, single stranded binding protein, and DNA ligase (Modrich, 1995)

1.2.6.2.3. The genetic bases of microsatellite instability in familial and sporadic colorectal cancer.

Discovery of the RER positive phenotype in sporadic and inherited colon cancer provided the first clue that defects in MMR system might play a role in colorectal carcinogenesis (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993; Inov *et al.*, 1993). Microsatellites are normally stable repetitive genetic sequences where the repeating unit is one to six bases (Weber *et al.*, 1989). Because of their repeating nature they are particularly prone to slippage during replication which results in a small loop in either the template or nascent DNA strand (Kunkel *et al.*, 1990). Despite these replicative mistakes which occur in all cells microsatellites are normally stable in length because of the efficiency of the MMR system. However, the RER+ cancers have lost strict maintenance of microsatellite length and their microsatellites appear unstable - they differ in length compared to microsatellites in normal tissue.

Originally it was not clear why instability of microsatellites, which are almost always non-coding, should contribute to carcinogenesis. This was explained by the discovery that RER+ colorectal cancers also exhibit an increased mutation rate in endogenous expressed genes (Bhattacharyya *et al.*, 1995; Eshleman *et al.*, 1995). Sequence analysis of the mutations in a selectable reporter gene showed the increased rate of not only the predictable frameshift mutations but also substantial numbers of base substitutions equal in number to the frameshifts (Bhattacharyya *et al.*, 1995; Eshleman *et al.*, 1996). In addition, genes such as *TGFβ RII*, which functions as a tumour suppressor gene and *BAX* - a significant effector in the initiation of apoptosis, contain homopolymeric repetitive sequences, a classical target site for nucleotide mismatch. In fact inactivating mutations in both *TGFβ RII* and *BAX* have been found to occur in colorectal cancers with microsatellite instability (Markowitz *et al.*, 1995; Parsons *et al.*, 1995; Akiyama *et al.*, 1996a; Togo *et al.*, 1996a; Rampino *et al.*, 1997; Ouyang *et al.*, 1998; Yagi *et al.*, 1998; Yamamoto *et al.*, 1998b).

Microsatellite instability is present in most tumours from patients with hereditary non-polyposis colorectal cancer where it is a consequence of mutations in one of the DNA mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3* or *hMSH6*) (Peltomaki *et al.*, 1993; Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Nystrom-Lahti

et al., 1994; Papadopoulos *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996; Akiyama *et al.*, 1997b; Miyaki *et al.*, 1997). The majority of HNPCC patients carry a germline mutation in either *hMSH2* or *hMLH1* (Liu *et al.*, 1996). RER+ phenotype is also detected in approximately 15-20% of sporadic colorectal cancers (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Wu *et al.*, 1994; Borresen *et al.*, 1995; Liu *et al.*, 1995; Bubb *et al.*, 1996; Eshleman and Markowitz, 1996; Konishi *et al.*, 1996). However, inactivating mutations in MMR genes appear to play a limited role in sporadic colorectal cancers with microsatellite instability. Recent studies suggest that inactivation of MMR system in sporadic tumours occurs through hypermethylation of CpG sites in the promoter region of *hMLH1* followed by loss of its expression (Cunningham *et al.*, 1998; Herman *et al*, 1998; Deng *et al.*, 1999; Maekawa *et al.*, 1999; Wheeler *et al.*, 1999).

Despite different molecular mechanisms underlying RER+ phenotype in familial and sporadic form, all colorectal cancers with microsatellite instability, tumours from HNPCC patients and sporadic cancers share certain clinicopathological characteristics. They are usually located in the proximal colon (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Lothe *et al.*, 1993; Aaltonen *et al.*, 1993; Kim *et al.*, 1994), they more frequently show poor differentiation (Ionov *et al.*, 1993; Lothe *et al.*, 1993; Kim *et al.*, 1994), mucin production (Kim *et al.*, 1994) and they have near-diploid DNA content (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993; Remvikos *et al.*, 1995; Schlegel *et al.* 1995). Sporadic RER+ colorectal cancers also exhibit a substantial survival advantage, independent of other prognostic factors (Lothe *et al.*, 1993; Thibodeau *et al.*, 1993; Bubb *et al.*, 1996).

1.2.6.3. Chromosomal instability in colorectal cancer.

The majority of sporadic colorectal cancers display a distinct phenotype characterised by an increased number of chromosomal abnormalities. Gains, losses and structural alterations within chromosomes consisting of chromosome deletions, inversions and gene amplifications are found in many colorectal tumours. Loss of genetic material from 1p, 5q, 8p, 17p and 18q, amplifications of 8q, 13q, 20q and duplication of chromosome 7 are among the chromosomal abnormalities most frequently identified

in colorectal carcinomas (Reichmann *et al.*, 1981; Muleris *et al.*, 1985, 1988, 1990, 1994; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Xiao *et al.*, 1992; Bardi *et al.*, 1993a, 1993b, 1995; Barletta *et al.*, 1993; Herbergs *et al.*, 1994; Gerdes *et al.*, 1995; Bomme *et al.*, 1996; Herbergs *et al.*, 1996; Ried *et al.*, 1996; Mertens *et al.*, 1997).

Abnormal chromosome copy number (aneuploidy) is nearly ubiquitous in cancer (Mertens *et al.*, 1997; Mitleman *et al.*, 1997). It could therefore be argued that it results simply from the abnormal structure and growth properties of cancer cells. However, there is increasing evidence for its association with underlying chromosomal instability (Lengauer *et al.*, 1997b; Cahill *et al.*, 1998). As defective mismatch repair drives neoplasia in MIN tumours and provides genetic variation for natural selection, CIN has also been suggested as an alternative pathway in colorectal carcinogenesis driving the tumorigenic process in the majority of colorectal tumours (Lengauer *et al.*, 1997b). While the mechanisms underlying microsatellite instability are known to involve the mismatch repair system (Peltomaki *et al.*, 1993; Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Papadopoulos *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996; Akiyama *et al.*, 1997; Miyaki *et al.*, 1997) and are relatively well understood, the molecular basis of chromosomal instability, present in the majority of malignancies, is just beginning to be explored.

A number of mechanisms that might underlie chromosomal instability have been suggested. It is suspected that, in contrast to MIN when only a few genes are responsible for the phenotype, there is a large number of genes which when altered can give rise to CIN. They include genes involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function and centrosome/microtubule formation and dynamics, as well as "checkpoint" genes that monitor the progression of the cell cycle (Langauer *et al.*, 1998).

Alterations in DNA methylation patterns have also been suggested to play a role in chromosomal instability. DNA methylation is involved in suppressing mitotic recombination and/or contributing to faithful chromosomal segregation during mitosis (Chen *et al.*, 1998). Global DNA hypomethylation has been shown to be associated with chromosomal aberrations including mitotic dysfunction and it has been frequently

observed in colorectal cancer cells where it has been associated with abnormal chromosomal structures (Lengauer *et al.*, 1997a).

Defects in p53 function have been implicated too in genome destabilisation. Abnormalities of p53 are known to strongly predispose towards chromosomal instability in many circumstances (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993; Deangelis *et al.*, 1993; Meling *et al.*, 1993; Carder *et al.*, 1995; Bouffler *et al.*, 1995; Donehower *et al.*, 1995; Gualberto *et al.*, 1998; Venkatachalam *et al.*, 1998). *In vitro* studies showed cells in culture often become grossly aneuploid at the same time that p53 is inactivated (Harvey *et al.*, 1993). It is unlikely, however, that p53 is generally responsible for CIN, as several cancer lines with p53 mutations are diploid and chromosomally stable (Lengauer *et al.*, 1997b, Eshleman *et al.*, 1998a). These findings indicate that, although p53 defects are unlikely to be a primary cause of chromosomal instability they probably contribute to this process.

Altered expression of certain spindle checkpoint genes can result in aneuploidy. For example decreased expression of *hMAD2* was observed in the T47D breast cancer cell line which failed to undergo mitotic arrest after nocodazole (mitotic spindle inhibitor) treatment. This suggests that loss of *hMAD2* function might lead to aberrant chromosome segregation and aneuploidy (Li and Benezra R, 1996). A small fraction of colorectal cancer cell lines, proved to exhibit CIN, have been shown to contain somatic mutations in mitotic checkpoint genes such as *hBUB1* and *hBUBR1* (Cahill *et al.*, 1998).

Several genes involved in the DNA-damage checkpoint have also been implicated to contribute to the CIN phenotype, including *ataxia telangiectasia mutated* (*ATM*) (Rotman and Shiloh, 1998), the *ATM*-related gene *ATR* (Smith *et al.*, 1998) and the *BRCA1* and *BRCA2* genes, which interact with the human *Rad51* homologue (Feunteun, 1998).

An abnormal number of centrosomes has been noticed in various human malignancies and suggested as a potential cause of CIN (Doxsey, 1998). Multipolar spindles have often been observed in human cancers but the molecular and genetic bases for the increased number of centrosomes have not yet been defined. Though the involvement

of certain genes such as ones encoding kinases aurora2/STK15 and PLK1 has been suggested (Bischoff *et al.*, 1998; Nigg, 1998; Zhou *et al.*, 1998).

Despite these clues the molecular basis of CIN in most human cancers remains unknown. The fact that genetic defects in so many genes can lead to CIN might explain why this phenotype is so common. Accordingly with so many genes involved, each one probably plays a role in a small proportion of cases (Lengauer *et al.*, 1998).

1.2.6.4. Summary: genetic models of colorectal carcinogenesis in sporadic colorectal cancer.

The following figures summarise the most important genetic events in colorectal carcinogenesis in MIN and CIN sporadic colorectal carcinomas.

Figure 3. *Genetic events in MIN pathway in sporadic colorectal cancers.*

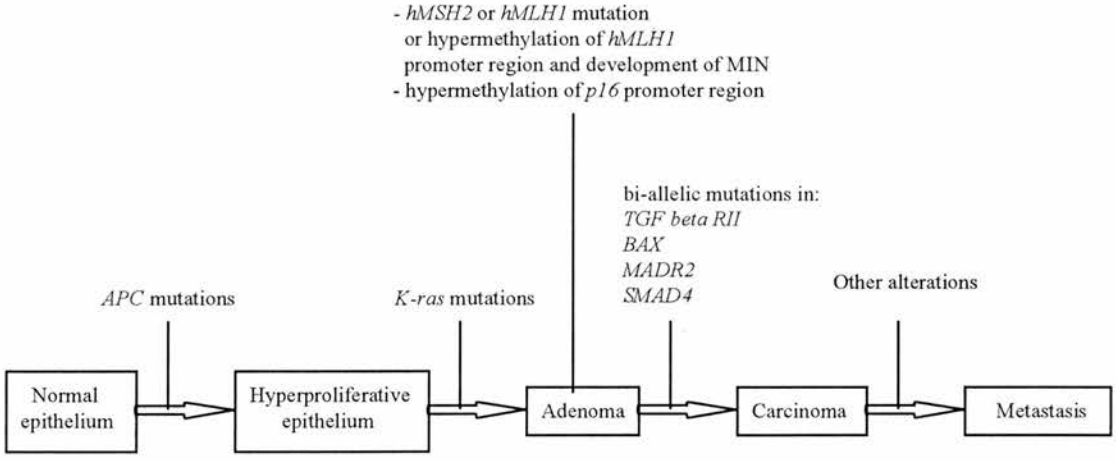
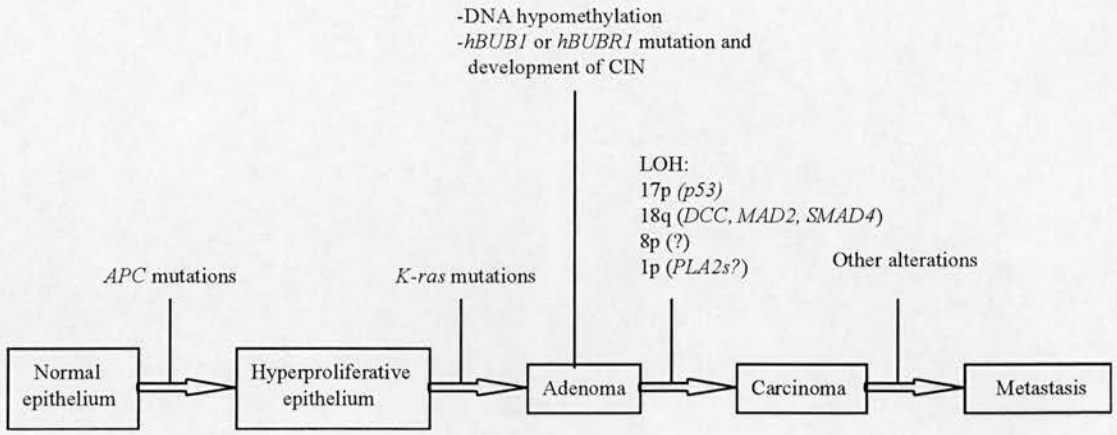


Figure 4. *Genetic events in CIN pathway in sporadic colorectal cancers.*



1.3. Aims of the study.

The aims of this project were to determine (i) the extent of intratumoral genetic variation in a series of sporadic colorectal cancers through sampling multiple sites of the primary tumour (spatial variation) and (ii) the evolution of genetic changes in colorectal cancer cells in time through their growth as xenografts in SCID mice (temporal variation). This study also aimed to determine (iii) the association of genetic changes detected within a tumour with two known types of genomic instability, (iv) the association of p53 defects with both types of genomic instability and (v) the extent to which colorectal cancer xenografts are representative of the primary tumour from which they are derived.

In order to do this samples of fresh tumour tissue were collected from multiple sites from a series of 22 sporadic colorectal cancers obtained from 21 patients. Colorectal cancer xenografts were established in SCID mice from primary tumour samples taken from multiple sites. Successfully established xenografts were harvested and fresh tissue obtained for analysis. Four loci, D2S123, D13S160, the poly (A) tract BAT-26 and the $(A)_{10}$ repeat in exon 3 of *TGFβ RII* were examined in all primary tumour and xenograft samples to determine their RER status. The presence of chromosome copy number changes was examined in all samples by Comparative Genomic Hybridisation. Additionally tumour cells' total DNA content was confirmed by Flow Cytometry

analysis. The immunohistochemical analysis of stabilised p53 protein was carried out and was complemented by the mutation analysis of *p53* gene in exons 5-8.

Specific genetic changes detected by methods employed at different sites within primary tumours were compared to determine the level of genetic intratumoral heterogeneity and whether single sample analysis could affect the results of such study. These specific genetic changes were compared with changes detected in the corresponding colorectal cancer xenografts in order to determine whether and to what extent they are genetically representative of the primary tumour. Multiple sample analysis also allowed to determine whether both groups of sporadic colorectal cancer exhibit specific patterns of chromosomal changes possibly selected for during tumour development and whether these patterns are similar or differ in these tumour groups.

The results of this study provided valuable new information on genetic intratumoral heterogeneity in sporadic colorectal cancer and its association with different mechanisms of underlying genomic instability. Although this study did not address directly the issue of response to therapy, the data gathered is important in establishing whether classification of genetic instability in this way has a bearing on response to different therapeutic agents.

CHAPTER 2.

Materials and methods.

2.1. Tissue specimens.

Fresh tissue samples were obtained from 21 colectomy specimens containing 22 colorectal carcinomas removed at operation between April 1997 and November 1997. One colectomy specimen contained two primary synchronous adenocarcinomas both of which were used for the study and treated as separate tumours (tumour No9 and 9'). Specimens were immediately collected from the operating theatre and delivered on ice to the Department of Pathology at the University of Edinburgh. Blocks of fresh tissue (approximately 10x5x5mm) were taken from 2 to 4 different sites (depending on the size of the tumour) from each colorectal cancer and one from the normal mucosa at a point distant from the lesion. Each block of tissue collected from the tumour was subsequently divided into three separate pieces. One portion was placed in 1.5ml screw-capped vial, snap frozen in liquid nitrogen and stored at -70°C awaiting DNA extraction and flow cytometry analysis. The middle portion was fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD), paraffin-processed and sections were stained with haematoxilin and eosin (H&E) following standard methodology for histological assessment. Sections from paraffin blocks were also used for immunohistochemical detection of stabilised p53 protein.

2.2. Establishment of colorectal cancer xenografts in SCID mice.

The third portion of each of the fresh tissue blocks from the tumours was first washed in PBS and then placed in a vial with antibiotic medium (Glasgow medium supplemented with penicillin, streptomycin, HEPES and 10% serum) and immediately taken for implantation in SCID mice. Alternatively, in case when the implantation could not be performed immediately samples were placed in freezing medium (Glasgow medium/dimethyl sulphoxide) for slow freeze and stored at -70°C until animals were available for establishing xenografts.

Xenografts were established by implantation of two pieces of cancer tissue approximately 2x2mm through a small dorsal incision in severe combined immunodeficiency syndrome (SCID) mice. SCID mice are characterised by deficient lymphocyte B and T function. Their lymphocytes are unable to correctly rearrange the immunoglobulin and T-cell receptor genes which normally occurs by site-spacific [V(D)J] recombination (Bosma *et al.*, 1988). Thus these mice do not reject implanted foreign tissue. However they retain some non-specific macrophage-mediated immune response, useful for removal of bacterial contamination from implanted tumour tissue. Tumours were allowed to grow for a variable length of time usually until externally visible tumour bulk of approximately 1 cm in diameter was reached. This time varied extremely between tumours from 23 days to over 5 months but on average lasted 12 weeks. The mice were then killed and the xenograft tissue was harvested. Part of this tissue was snap frozen in liquid nitrogen for DNA extraction and flow cytometry analysis. Part of it was fixed, paraffin processed and stained with haematoxilin and eosin for the histological assessment. Paraffin sections were also used for immunohistochemical detection of stabilised p53 protein.

The total of 27 colorectal cancer xenografts were successfully established from 10 primary tumours.

2.3. DNA extraction.

2.3.1. Extraction of DNA from frozen tumour tissue, normal colonic mucosa and xenograft tissue.

The method of Goelz *et al.*, (1985a) was used for the DNA extraction. A small piece of frozen tissue was finely chopped with a scalpel in a petri dish and placed in 1.5 ml tube containing 0.5ml TE-9 SDS and 0.5mg/ml proteinase K. This mixture was incubated for 48 hours at 48°C and was occasionally shaken. After proteinase K digest was completed, an equal volume of TE-saturated phenol was added to the tube, mixed and centrifuged at 10000 x g for 2 minutes. The upper, aqueous layer was carefully removed to a new tube leaving behind any precipitates formed at the boundary between the two phases. An equal volume of PC-9 was added to it and the

tube content was mixed and centrifuged again. This extraction procedure was repeated using an equal volume of 24:1 chloroform:iso-amyl alcohol. The upper layer was once again removed to a new tube and 0.25ml 7.5M ammonium acetate was added. DNA was precipitated from the solution with 1ml of cold absolute ethanol and left -70°C for 1 hour. In order to obtain high molecular weight genomic DNA it was removed from the tube with a sterile pipette tip. DNA was briefly washed in 70% ethanol and resuspended in 150μl sterile TE buffer. It was allowed to dissolve at 4°C for at least 3 hours, but usually overnight before an OD reading to estimate the DNA concentration was taken.

TE-9 SDS:	**PC-9:**	**TE:**
500mMTris pH8	480ml phenol	10mM Tris.
20mM EDTA	320ml TE-9	1mM EDTA.
10mM NaCl	640ml chloroform	
1% SDS		

2.3.2. Estimation of DNA concentration.

DNA concentration was measured using CE 2020 spectrophotometer with readings of OD taken at 260nm. An optical density ratio (OD 260/280) was measured for each sample to estimate DNA purification. Concentration of DNA was adjusted to 100μg/ml for use as template in polymerase chain reactions.

2.4. Analysis of microsatellite instability.

2.4.1. PCR of microsatellite sequences.

Two dinucleotide repeat sequences, D2S123 and D13S160 (Gyapay *et al.*, 1994), the poly (A) tracts *BAT-26* (Hoang *et al.*, 1997) and the $(A)_{10}$ repeat in exon 3 of *TGFβ RII* were analysed for evidence of microsatellite instability. Primer sequences are given in Table 2. All four loci were examined in each of the 74 samples collected from 22 primary colorectal cancers and in all 27 xenografts. Reactions were carried out in 0.5 ml tubes in 50μl volumes with a final concentration of 1xPCR buffer,

200μM each dNTP, 0.5μM each primer and 300ng genomic DNA template for all loci except *BAT-26* where a final concentration of 100μM of each dNTP with 450ng genomic DNA template was found to yield better results. DMSO was added to each reaction to a final concentration of 10% to improve the reaction specificity. Table 2 contains details of magnesium ion concentrations and annealing temperatures for each primer set. One drop of paraffin oil was added to each tube to prevent evaporation. Reactions were hot started by adding 1.25 U *Taq* DNA polymerase to each tube after denaturing the samples at 94°C for 5 minutes. 35 cycles of amplification were carried out on a thermal cycler with each cycle consisting of 30 seconds denaturation at 94°C, 30 seconds annealing at variable temperature (Table 2) and 30 seconds extension at 72°C. Reactions' products were always checked on a 2% agarose gel (See 2.4.2).

PCR buffer (Buffer IV, Advanced Biotechnologies Ltd):

750mM Tris-HCl (pH9 at 25°C)

200mM $(NH_4)_2SO_4$

0.1% w/v Tween

Table 2. *Primer sequences for amplification of microsatellites, BAT-26 and TGF-β RII.*

Locus	$[Mg^{2+}]$ conc.	Annealing temp.°C	Approximate allele size (bp)	Primer sequences (5'-3')
D2S123	2mM	58	210-240	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC
D13S160	1.5mM	58	229-241	CGGGTGATCTAAGGCTTCTA GGCAGAGATATGAGGCAAAA
BAT-26	1.5mM	50	134	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACCC
TGFβ RII Ex3	4mM	58	267	CCTCGCTTCCAATGAATCTC TTGGCACAGATCTCAGGTCC

2.4.2. Agarose gel electrophoresis.

2g electrophoresis-grade agarose was added to 100ml 1xTBE buffer, mixed and heated to boiling in a microwave oven. The mixture was stirred every 30 seconds, to dissolve the agarose. The gel was allowed to cool before 20 ng/ml ethidium bromide was added and it was poured into a 12x16cm gel mould with a 20-well comb. PCR products were checked by loading 5µl of the PCR product mixed with 1µl loading dye into the wells of the agarose gel. Electrophoresis was carried out at 125V in 1xTBE buffer until the bromophenol blue dye migrated approximately 5cm through the gel. The PCR products were run against 500ng of marker V (Boehringer-Mannheim Ltd) to confirm the size. Gels were visualised on a Gel Doc (BIO RAD) and usually a photograph was taken at the same time.

10xTBE:

107.8g Tris (0.89M)

55g Boric acid (0.89M)

7.44g Na_2EDTA (0.02M)

in 1l DDW

pH adjusted to 8.3

Loading dye:

30% glycerol

0.25% bromophenol blue

0.25% xylene cyanol FF

2.4.3. Radioactive isotope labelling of primers.

The 5' end of one of the pair of primers for each locus was labelled with γ^{33}PdATP using T4 polynucleotide kinase. 4µl of 5xT4 kinase buffer was mixed with 7µl of DDW in a microfuge tube. 5µl of diluted primer (10µM), 2µl of γ^{33}PdATP (equivalent to 20µCi) and 2µl (20U) of T4 polynucleotide kinase was added giving a final volume of 20µl and the reaction was placed in a water-bath at 37°C for one hour. This amount was sufficient for labelling up to 40 samples.

Labelled primers were incorporated into PCR products by running 5 cycles of PCR under conditions specified for each locus (see Table 2). The reaction mixture consisted of a final concentration of 1xPCR buffer, 175µM of each dNTP, 1.5mM $MgCl_2$, 3.75mM of end labelled primer to which 10µl of the appropriate PCR product

was added to the final volume of 50μl. 1.25U of *Taq* DNA polymerase was added to each reaction and the mixture was covered with one drop of mineral oil.

2.4.4. Preparation of polyacrylamide denaturing gel.

Amplified microsatellite sequences for loci D2S123 and D13S160 and poly (A) *BAT-26* sequence were run on polyacrylamide denaturing gel to detect shifts in electrophoretic mobility between tumour and normal DNA sequences.

34x41cm glass plates for vertical gel electrophoresis were cleaned with cream cleaner, well rinsed and wiped with 70% ethanol. One plate was wiped with 1ml Gel Slick (AT Biochem), left for 5 minutes and cleaned with 70% ethanol. 0.4mm spacers and 32 square-well comb were used. The gel was prepared by adding 50μl of TEMED and 500μl freshly prepared (less then two weeks old) 10% ammonium persulphate to 75ml acrylamide solution immediately before pouring the gel. The gel was left to set for at least half an hour. The combs were removed, the wells rinsed with 0.5x TBE and the gel was preran at 70W in 0.5x TBE for one hour prior to loading the samples in order to raise its temperature to about 55°C.

Acrylamide solution:

11.25ml 40% Acrylamide (19:1 acrylamide:bis)
'Instagel' (Severn Biotech Ltd)
31.5g urea (7M)
3.75ml 10xTBE
36.25ml DDW
stored protected from light at 4°C

0.5 x TBE buffer:

5.39g Tris (0.04M)
2.75g Boric acid (0.04M)
0.372g Na_2EDTA (0.001M)
in 1l DDW
pH adjusted to 8.3

2.4.5. Electrophoresis on polyacrylamide denaturing gel.

Ten microlitres of $\gamma^{33}P$ labelled PCR product was denatured at 95°C for 5 minutes in 0.5x volume short tandem repeat (STR) loading solution then immediately placed on ice. The wells of the polyacrylamide gel were thoroughly rinsed with 0.5xTBE and then 7.5μl of denetured PCR product was loaded into each well. The electrophoresis was carried out at 70W for 1-2 hours depending on the size of the PCR product, but

usually the gel was run until the xylene cyanol marker in the loading dye reached the bottom of the gel. Optimal running temperature was 50-55°C.

STR loading solution:

10mM NaOH

95% formamide

0.05% bromophenol blue

0.05% xylene cyanol

2.4.6. Electrophoresis on MDE gel.

The $(A)_{10}$ sequence in exon 3 of TGF-β RII was assessed using Single Stranded Conformational Polymorphism analysis (SSCP). MDE gel was prepared as described in 2.8.3. Ten microlitres of ^{33}P-labelled PCR product was denatured for 5 minutes at 50°C in 2μl of the denaturing solution. Six microlitres of stop solution was added to each tube and the samples were placed on ice and then loaded quickly onto the gel, after rinsing the wells with 1xTBE. Electrophoresis was carried out in 1xTBE buffer at 6W overnight until the xylene cyanol dye reached the bottom of the gel.

Denaturing solution:

0.5M sodium hydroxide

10mM EDTA

Stop solution:

95% formamide

20mM EDTA

0.05% bromophenol blue

0.05% xylene cyanol

2.4.7. Detection and assessment of electrophoretic mobility of microsatellite sequences, BAT-26 and TGFβ RII.

Gels were fixed in a solution of 10% methanol and 10% acetic acid, transferred to Whatman paper No17 and dried on a vacum gel drier. Autoradiography was carried out for 1-3 days and autoradiographs were assessed visually for the presence of shifts in electrophoretic mobility of amplified sequences from tumour samples compared to normal DNA.

2.5. Analysis of imbalanced chromosomal abnormalities using Comparative Genomic Hybridisation.

A series of 74 samples collected from multiple sites from 22 primary colorectal carcinomas and 26 samples obtained from successfully established xenografts derived from 9 primary tumours were analysed for the presence of unbalanced chromosomal abnormalities using Comparative Genomic Hybridisation (CGH). CGH is a method that detects changes in chromosome copy number (whole or partial) through competitive hybridisation of DNA derived from normal and tumour tissue onto a normal human chromosome metaphase spread (Kallioniemi *et al.*, 1992).

2.5.1. Preparation of normal human metaphase spreads.

2.5.1.1. Blood culture.

Fresh blood, approximately 10 ml, was collected from a healthy male volunteer into a lithium heparin tube. Using sterile technique culture medium was prepared. It consisted of RPMI medium (Dutch modification), supplemented with 10% foetal bovine serum (heat inactivated), phytohemagglutinin (final concentration 9μg/ml) and L-glutamine (final concentration 2mM). The medium was filtered through a 0.2μm filter and aliquoted into 8 flat-bottomed 25cm^2 plastic tissue culture flasks, 10ml in each. 0.8 ml of blood was added into each flask, swirled to mix, and cultures were incubated at 37°C for 72 hours. One hour before harvesting (at exactly 71 hours), 100μl of 10μg/ml colcemid was added and the incubation continued for another hour. After exactly 72 hours the cultures were transferred into 10ml centrifuge tubes and spun at 800 x g for 5 minutes to pellet cells. Then the supernatant was removed, the pellet resuspended in prewarmed (37°C) 0.075M KCl hypotonic solution and incubated in a 37°C waterbath for 10 minutes. The tubes were removed from the bath, centrifuged again at the same speed and then the supernatant was removed and the pellet tapped to loosen the cells. Cells were carefully fixed by adding very slowly drops of fresh ice-cold fixative solution, consisting of 3:1 methanol:glacial acetic acid to a final volume of 5ml while being gently vortexed. Then the tubes containing fixed cells were placed at -20°C for one hour and spun at 800 x g for 5 minutes. In order to

obtain a white pellet of cells at the bottom of the tube, the procedure of fixing and spinning was repeated twice. Fixed cells could be stored in 5ml of fixative solution at -20°C for up to a year.

2.5.1.2. Preparation of metaphase chromosome spreads.

Selected microscope slides were prepared by soaking overnight in 10% Decon detergent and rinsing in water for about an hour. Then the slides were transferred to 100% ethanol to which a few drops of concentrated hydrochloric were added. They could be stored in this solution until they were used. The tubes with previously prepared metaphase preparations were centrifuged at 800 x g for 5 minutes in order to pellet the cells. The supernatant was then removed and the cells were resuspended in a small amount (a few drops) of freshly prepared fixative solution. The amount of fixative solution required varied and depended on the size of the pellet. Usually the fixative solution was added dropwise until resuspended cells formed a milky solution. Slides were removed from ethanol, polished with a soft cloth and just before use placed at -20°C for 2 minutes to facilitate spreading of chromosomes. One drop of fixed chromosome preparation was dropped from approximately 50 cm height directly on top of each slide. A diamond pencil was used to mark the position of the drop on a slide. Before the slides were used in the hybridisation procedure they were checked on a phase contrast microscope at x40 magnification to ensure the sufficient number of quality metaphases (with chromosomes of the right length and with few overlapping ones) was present on each slide.

The slides were stored at room temperature in a vacuum desiccator for at least 1 week, but not longer than 2 weeks, before they were used for setting up hybridisation.

2.5.2. Labelling of control and test DNA by nick translation.

Test DNA was extracted from frozen tissue according to the method of Goelz *et al.* (1985a) (see section 2.3).

DNA obtained from lymphocytes of two healthy male volunteers was used as control DNA for counter-hybridisation. This was kindly provided by Dr Lucy J Curtis who

also confirmed the normal karyotype of the lymphocytes from which the control DNA was extracted.

Incorporation of digoxygenin-11-dUTP (normal) or biotin-16-dUTP (test samples) by nick translation was used to label normal male DNA and test DNA obtained either from a primary tumour or a xenograft. DNA fragments' length of between 300 and 3000kb, as assessed by running double-stranded DNA on 1% agarose gel against DNA mass ladder (Life Technilogies Ltd) were found to provide optimal hybridisation in this study. Using shorter fragments resulted in chromosomes appearing fuzzy and if fragments were too large hybridisation was rather poor.

The nick translation reaction mixture was prepared. First DNase I (10u/µl) was diluted 1/15000 in ice-cold DDW and placed on ice. One 0.5ml microfuge tube for each sample was also placed on ice to cool. The following components were added to each tube:

2µg of genomic DNA, either normal male or test sample

100µM dATP

100µM dGTP

100µM dCTP

80µM dTTP

40µM digoxygenin-11-dUTP (control) or biotin-16-dUTP (test sample)

1x nick translation buffer

DDW to 38µl

1µl (10U) DNA polymerase I

1µl diluted DNase I

The components were gently mixed, briefly centrifuged and then the mixture was incubated at 16°C for 1 hour. After the incubation, in order to check the size of DNA fragments, the tubes were placed on ice and 5µl of the mixture was removed and run against 1kb ladder molecular weight marker (Life Technologies Ltd) on a 1% agarose gel until the bromophenol blue had migrated approximately 6cm through the gel. If there were fragments bigger than 3kb, the tubes were placed again in the water-bath and the incubation at 16°C continued for another 10-20 minutes after a further 1µl DNase I was added to the reaction tube. When the desirable fragments' length was

reached, which was between 300 and 3000 bp long, 2µl 0.2mM EDTA/1%SDS were added to stop the reaction. Fine sephadex G50 Quick Spin columns were used to clean the labelled DNA and to remove the remaining unincorporated nucleotides (Boehringer Mannheim Ltd) from the mixture. The column was first inverted to resuspend the sephadex and then spun at 6000 x g for two minutes to dry. Nick translated DNA was added to the top of the column and it was spun at 6000 x g for 4 minutes and the drops collected in a tube. Concentration of DNA was recalculated, making adjustment for the mixture volume obtained after cleaning through the column. Labelled DNA was stored in -20°C until use.

10x nick translation buffer:

0.5M Tris pH 7.5

0.1M $MgSO_4$,

1mM dithiothreitol

500µg/ml bovine serum albumin fraction V.

2.5.3. Hybridisation.

The optimal hybridisation conditions were previously established by Dr Lucy J Curtis. A modification of the method of Kallioniemi *et al.* (1992) with incorporated changes from the methods of Verma and Babu (Verma and Babu, 1995) and from a method provided by Dr H.Morrison at the Human Genetics Unit, MRC, Edinburgh was found to work most consistently.

2.5.3.1. Probe preparation.

The probe mixture consisted of 500ng of normal male genomic DNA labelled with digoxygenin mixed with 500ng of biotin-labelled test DNA and 10µg human Cot-1 DNA. 100µl of ice cold ethanol was added to each tube containing the probe mixture in order to precipitate DNA. The tubes were left at -70°C for 1 hour and then the ethanol was removed by placing the tubes in a vacuum centrifuge and spinning them at 1000 x g. Precipitated probes could be stored at -20°C until they were needed.

Prior to hybridisation the probes were resuspended in 7.5µl CGH buffer. The mixture was vortexed and left to dissolve at room temperature for at least one hour. In order to denature the probes, 7.5µl deionised formamide was added, tubes were vortexed, briefly centrifuged and placed for 5 minutes in a water-bath at 70°C. To enhance the blocking effect of non-specific sequences by human Cot-DNA, after denaturation and prior to the hybridisation step, the probes were allowed to reanneal for between 30 minutes and 2 hours at 37°C.

Slide preparation (see below) was carried out simultaneously to the probe preparation.

CGH buffer:

20% dextran sulphate (diluted from autoclaved 50% stock)

4xSSC see 2.9.4

2.5.3.2. Slide preparation.

Competitive hybridisation of test and normal labelled DNA prepared as above (2.5.2) was carried out on normal male metaphase chromosome spreads previously prepared and stored for minimum 1 week (see section 2.5.1.2). The preparation was carried out in 50ml-capacity Coplin jars with forceps being used for moving the slides. 200ml-capacity glass staining dishes with slides being placed in a slide rack were used for ethanol washes.

In order to remove RNA slides were first treated with RNase. They were placed in 10µg/ml RNase A solution at 37°C for 1 hour followed by a wash in 2xSSC at room temperature for 2 minutes. Slides were then washed in proteinase K buffer for 2 minutes and placed in 100ng/ml proteinase K solution for 2.5 minutes, followed again by 2 minute wash in proteinase K buffer. They were then dehydrated through 70%, 90% and 100% ethanol for 2 minutes in each. Slides were warmed on a hotplate to 70°C prior to being placed into 70% formamide solution at 70°C for 3 minutes. This procedure was carried out in a fume cabinet and a thermometer was used to check whether the formamide temperature was exactly 70°C as required, before the slides were placed there. This procedure was always carried out in a Coplin jar, and no more

than 4 slides at a time were added at one time. This was necessary to maintain a constant solution temperature, which was essential for successful slide preparation. After denaturation, slides were plunged immediately into 70% ice-cold ethanol and kept on ice for 5 minutes. They were then dehydrated again through the ethanol series as before.

Slides were marked and warmed up on a hotplate at 37°C prior to adding the probe. Alongside small ($20mm^2$) coverslips which previously had been thoroughly cleaned with ethanol were also warmed up. The probe was removed from the water-bath were it was kept at 37°C and placed onto a cover slip. Appropriate steps were taken to ensure that the temperature did not drop below 37°C. The slide was placed chromosome-side down on top of the coverslip containing the probe. Rubber solution was used to seal the coverslip with the slide to prevent the evaporation of the hybridisation mixture. The slides were then placed in a humidified chamber and incubated at 37°C for 2-3 days.

RNase A solution:	**Proteinase K buffer:**	**Proteinase K solution:**
10µg/ml RNase A in 2xSSC	0.02M Tris pH 7.5 0.002M calcium chloride	100ng/ml proteinase K in proteinase K buffer

Formamide solution:	**Deionised formamide:**
35ml deionised formamide 2xSSC	500ml formamide added to 25g ion-exchange resin beads, stirred for 45 minutes, then filtered through a paper filter. Stored at -20°C.

2.5.4. Detection of labelled probes.

After the slides were incubated for 48-72 hour they were removed from the humidified chamber. The rubber sealant was carefully removed and an attempt was made not to tear off the coverslip from the slide. The slides were then washed for 3 minutes in 50% formamide/2xSSC at 45°C, allowing the cover slips to float off. This procedure was repeated three times and followed by four 3 minutes washes in 2xSSC at 45°C and then four 3 minutes washes in 0.1xSSC at 45°C. Prior to the addition of

the antibody solution, slides were washed for 2 minutes in 0.1% Tween 20/4xSSC at room temperature and then they were incubated with 100µl blocking buffer under a clean large cover slip at room temperature for 5 minutes. Then the coverslips were removed and the slides were drained. One houndred µl fluorescein/rhodamine solution was added to each slide and the slides were covered by a new clean cover slip. They were incubated for 30 minutes in a damp chamber in the dark at 37°C. Slides were then drained and washed three times for 2 minutes in 0.1% Tween 20/4xSSC at 37°C, drained again and air dried. 35µl DAPI/antifade were mounted onto a large coverslip which was placed on top of the slide. The slides were then sealed with nail varnish, and left overnight in the dark at 4°C before the CGH analysis was carried out.

Blocking buffer:

0.5g skimmed milk
2ml 20xSSC
made to 10ml with DDW and heated to 50°C to dissolved, aliquoted into 1.5ml tubes and spun at 10000 x g for 15 minutes at 4°C.

Fluorescein/rhodamine solution:

1ml blocking buffer
5µg/ml fluorescein-avidin DCS
Spun at 10000 x g for 15 minutes at 4°C prior to addition of 10µg/ml anti-digoxygenin rhodamine

DAPI/antifade solution:

0.75µg/ml DAPI in Vectashield antifade solution

2.5.5. CGH analysis.

The Apple MacIntosh-based Quantitative Image Processing System (QUIPS) software (Vysis, UK, Ltd) was used to assess and analyse the hybridisation. The system consists of a fluorescent microscope (Zeiss Axioskop 20) with an attached CCD camera (SenSys) and a triple bandpass filter. The filter is set for three fluorochromes used in the method: rhodamine, fluorescein and DAPI (see Table 3). The microscope is also equipped with a computer-controlled filter wheel for image acquisition.

Slides were screened for good quality metaphases showing smooth hybridisation of both test and control DNA. Then grey-level images were captured at x1000 magnification. Each one of the three fluorochromes was excited separately by using a single band-pass excitation filter. The images captured were overlaid and displayed on the computer screen in pseudo-colour corresponding to the colour of each fluorochrome. A single grey scale DAPI image representing chromosome pseuo-G-banding pattern was used for chromosome identification. At least five metaphases were analysed from each slide. The QUIPS software generated green/red ratios for all chromosomes after overlapping and damaged chromosomes were excluded and then an average profile was calculated for each one of the 22 chromosomes included in the analysis. Green/red ratios generated from a minimum of 5 autosomes had to be included in the average ratio profile of each chromosome. Because sex chromosomes might be under different selection pressure in tumours derived from males and females they were excluded from the analysis in this study. The threshold at which chromosome copy number changes were scored was established at the green/red ratio of 1.125 and 0.875. At this cut-off point chromosome copy number changes could still be easily seen. It represents a loss or gain of one chromosome in 25% of cells in a diploid karyotype. The standard deviation of green/red ratio profiles generated for each chromosome was checked and slides were only used if it was small. Telomeres, pericentric regions and heterochromatic regions were excluded from analysis. A normal control (hybridisation of differently labelled normal DNA) was carried out with every batch of slides for quality control of hybridisation conditions. Chromosome 19 occasionally yield aberrant results. These results are included in the study but should be interpreted cautiously.

It should be pointed out that a substantial proportion of this study was carried out on primary colorectal cancers. The tissue used for DNA extraction, although not microdissected, was always histologically examined to confirm the presence of sufficient amount of tumour tissue. DNA extracted from these samples was therefore consistently contaminated to some degree with normal DNA. This would usually decrease the threshold at which chromosomal abnormalities could be detected. However, it did not present a problem and the 1.125/0.875 green/red ratio cut-off

point (which was previously applied in this lab for the CGH analysis of colorectal cancer xenografts by Dr Lucy J Curtis) was used for scoring chromosome copy number in the primary tumours as well as in the xenografts.

Table 3. *Characteristics of fluorochromes used in CGH.*

Fluorochrome	Optimal excitation λ (nm)	Optimal emission λ (nm)	Colour
4'-6-diamidino-2-phenylindole (DAPI)	355	450	Blue
Fluorescein	490	520	Green
Rhodamine	545	575	Red

2.6. Flow cytometry.

Flow cytometry was used for assessing cells' total DNA content.

2.6.1. Preparation of tissue.

The method of Vindelov *et al.* (1983) was followed for tissue preparation before the assessment of the DNA content was carried out on a Coulter EPICS-XL flow cytometer. A small fragment of frozen tissue, approximately $4mm^3$, was finely chopped using a sterile scalpel. The tissue macerate was suspended in 200μl of citrate buffer and 450μl of solution A was added to 100μl of this solution to obtain partial cell lysis. This mixture was mixed gently for 10 minutes at room temperature. Then 325μl of solution B was added to neutralise trypsin and remove RNA, followed by 10 minutes incubation at room temperature. After the incubation 250μl of solution C, containing propidium iodide (a DNA binding fluorochrome) was added. This procedure was carried out on ice and samples were left there for 10 minutes. The cell suspension was then filtered through a syringe with glass wool to dispose of large clumps of cells. The analysis was performed within 4 hours, during which time samples were kept on ice.

Solution A:	**Solution B**	**Solution C:**
15mg trypsin	250mg trypsin inhibitor	208mg propidium iodide
500ml stock solution	50mg ribonuclease A	500mg spermine tetrahydrochloride
pH7.6	500ml stock solution	500ml stock solution
	pH7.6	pH7.6

Citrate buffer:	**Stock solution:**
85.5g sucrose	2g trisodium citrate
11.76g trisodium citrate	121mg Tris
dissolved in 800ml DDW	1.044g spermine tetrahydrochloride
50ml DMSO added and volume made up to	2ml Nonidet P40
1litre with DDW, pH 7.6.	in 2l DDW, pH 7.6

2.6.2 Flow cytometry analysis.

This was carried out on a Coulter EPICS-XL flow cytometer. The excitation wavelength was 488nm. Immuno-Check alignment fluorospheres (Coulter Electronics Ltd) were used each time to check the alignment of the laser before the analysis of a sample batch begun and half-peak coefficients of variation were less than 2%. Cells were analysed according to forward and side scatter and double compensation was performed. At least 5,000 nuclei were analysed in each sample, and at the same time identical analyses of normal tissue from the same patient were carried out. The first peak was treated as a diploid one, and the DNA index of an aneuploid peak was calculated as a ratio of the aneuploid and the diploid peak position values.

2.7. Immunohistochemical detection of stabilised **p53** *protein.*

Immunohistochemical (IHC) methods are widely used to detect stabilised p53 protein. This is possible due to the short half-life (20-30 minutes) of the wild-type p53 protein, which is therefore undetectable by such methods (Oren, 1985). In this study DO-7 antibody (Dako), which recognises an amino terminal epitope of p53 protein and

reacts with both wild-type and mutant protein was chosen for the immunocytochemical detection of stabilised p53 protein.

Slides were prepared in the Department of Pathology, University of Edinburgh. 3μm sections from tissue fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax were cut and placed on poly-L-lysine (Sigma) coted slides.

Slides were first warmed up on a hot plate at approximately 50°C to allow the wax to melt and then they were deparaffinised by placing in xylene for 10 minutes. The sections were rehydrated through the washes in absolute ethanol for 1 minute, 74OP for 1 minute and 64OP for 1 minute and then they were rinsed in water. Slides were treated with 3% hydrogen peroxide for 15 minutes in order to block the endogenous peroxidase activity and then washed for 5 minutes in DDW and 5 minutes in tris-buffered saline (TBS). Slides were then wiped to remove excess fluid and the area around the section was marked with a wax pen to retain solutions. Sections were then incubated for 20 minutes with normal rabbit serum (NRS) diluted 1:5 in TBS then drained. The slides were incubated overnight at 4°C with a 1:100 dilution of DO-7 antibody in 1:5 NRS/TBS solution.

Following the overnight incubation, the slides were washed twice for 5 minutes in TBS before the secondary antibody was applied. The 1:400 dilution of biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd) in the above NRS/TBS solution was prepared and 100μl of this solution was added to each slide and incubated for 30 minutes at room temperature. An avidin/biotinylated horseradish peroxidase (HRP) complex (ABC kit, Dako Ltd) was prepared in the meantime by adding 1 drop of avidin and 1 drop of biotinylated horseradish peroxidase to 5ml TBS and allowing 30 minutes for the reagents to conjugate. The slides were washed twice in TBS for 5 minutes and 100μl ABC complex was added followed by 30 minutes incubation at room temperature. Then again slides were washed twice for 5 minutes in TBS and then incubated for 3 minutes in diaminobenzidine (DAB) solution at room temperature to allow formation of brown colouration through oxidation of DAB by HRP. This was followed by a 5 minutes wash in DDW and then slides were counterstained with haematoxylin for a few seconds. Haematoxylin was washed off

with tab water and the slides were dehydrated through alcohol washes: 1 minute in 64OP, 1 minute in 74OP, 1 minutes in absolute ethanol and then placed for 10 minutes in xylene. They were mounted with DPX mountant and examined on a light microscope. The presence, intensity and pattern of staining was noted for each slide. They were classified as positive if more than 10% of nuclei showed strong brown coloration.

PLPD:

0.1M lysine

0.1M periodate

2% paraformaldehyde

made to 50ml with Sørensen's phosphate buffer (0.05M, pH7.4).

5% potassium dichromate in 50ml DDW added immediately before use

TBS:

0.05M Tris/HCl

0.15M NaCl

pH 7.6

DAB solution:

5mg DAB

4.8ml DAB buffer

100µl 1:30 hydrogen peroxide solution

DAB buffer:

24ml 02.M Tris

38ml 0.1N HCl

0.0681g imidazole

DDW to 100ml

pH adjusted to 7.6

2.8. Mutation analysis of the p53 gene.

In order to exclude the possible presence of mutation not detectable by IHC mutation analysis of the *p53* gene was performed on 15 tumours with negative or weak immunohistochemical staining or staining present in less than 10% of cells. Studies previously carried out, determining p53 status at different tumour sites (Carder *et al.*, 1995) indicated that the mutation in *p53* gene, if present, occur at the adenoma/carcinoma interface during tumour progression and therefore can be readily detected in any part of the carcinoma. This is why originally only one representative

sample from each tumour was selected to be screened for the presence of mutation in *p53* gene. The mutation analysis was carried out using single-stranded conformational polymorphism analysis (SSCP) of exons 5-8, in which 90% of all mutations are located (Levine *et al.*, 1991). The four samples with known mutations in each of the exons 5-8 were included as positive controls as well as samples of normal DNA as negative controls.

2.8.1. PCR of p53 exons 5-8.

PCR was carried out using the primers listed in Table 4. The reaction mixtures of the total volume of 50µl consisted of 200ng genomic DNA with final concentration of 0.5µM of each primer, 200µM of each dNTP, 1.5mM $MgCl_2$ and 1x PCR buffer solution. One drop of paraffin oil was overlaid to prevent evaporation. This was heated to 95°C for 5 minutes and 1.25U of thermostable DNA polymerase was added to 'hot start' the reaction. 25 cycles of amplification were carried out in a thermal cycler with each cycle consisting of 30 seconds denaturaion at 94°C, 30 seconds annealing et variable temperature (Table 4) and 1 minute extention at 72°C. Then 5µl of diluted end labelled primer (see 2.8.2) was added to each reaction, tubes were spun, and further 10 cycles were carried out at the conditions described above.

Table 4. *Primer sequences for amplification of p53 exons 5-8.*

p53 exon	Primer sequence (5'-3')	Annealing temperature
5	TTCCTCTTCCTACAGTAGTC CCCAGCTGCTCACCATCG	55°C
6	CCTCACTGATTGCTCTTAGG AGTTGCAAACCAGACCTCAG	58°C
7	TGTGTTATCTCCTAGGTTGG TGGCAAGTGGCTCCTGAC	58°C
8	TCCTATCCTGAGTAGTGGT TCCTGCTTGCTTACCTCG	58°C

2.8.2. Radioactive isotope labelling.

In this reaction 5' end of one of the pair of primers for each exon was labelled with γ^{33}PdATP using T4 polynucleotide kinase. Primer sequences were diluted with sterile destilled water before use to a final concentration of 10ng/μl.

The reaction was carried out in a microfuge tube where 4μl of 5xT4kinase buffer was mixed with 7μl of DDW. 5μl of diluted primer, 2μl of γ^{33}PdATP (equivalent to 20μCi) and 2μl (20U) of T4 polynucleotide kinase was added giving a final volume of 20μl and the reaction was placed in a water-bath at 37°C for one hour. This amount was sufficient for labelling up to 40 PCR products and was diluted with DDW to the final volume sufficient to add 5 μl of the mixture to each of the PCR reactions.

5xT4 polynucleotide kinase buffer:

350mM Tris-HCl pH 7.6

50mM $MgCl_2$

500mM KCl

5mM 2-mercaptoethanol

2.8.3. Preparation of MDE gel.

0.5xMDE gel solution was prepared by mixing 36.5ml DDW, 3.6ml 10xTBE buffer, 6ml glycerol and 15ml MDE (AT Biochem). 256μl of freshly prepared 10% ammonium persulphate and 31μl TEMED were added, the solution was mixed and poured immediately between 34x41cm vertical electrophoresis plates with 0.4mm spacers and 49-well combs. The gel was allowed to polymerise at room temperature for one hour.

2.8.4. SSCP analysis of ^{33}P labelled PCR product.

1μl of denaturing solution was added to five microlitres of ^{33}P labelled PCR product. The mixture was denatured for 5 minutes at 50°C. Then 3μl of stop solution were added to each tube and the samples were placed on ice and loaded quickly onto the gel, after rinsing the wells with 1xTBE. Electrophoresis was carried out in 1xTBE

buffer at maximum 30W in order to keep the gel temperature at approximately 20°C. Running time varied according to fragment size, good fragment separation was achieved when samples were ran such that the xylene cyanol marker dye had migrated between 25 and 35cm through the gel.

After the electrophoresis was completed, the gel was transferred onto Whatman No 17 paper and dried on a vacuum gel drier. Autoradiography was carried out for 1-3 days and autoradiographs were assessed visually for shifts in electrophoretic mobility of amplified sequences compared to positive controls (samples with known mutations in exon 5-8) and negative controls (samples of normal DNA).

0.5xMDE gel solution:	**Denaturing solution:**	**10xTBE:**
15ml MDE gel	See 2.4.6	See 2.4.2
6ml glycerol		
3.6ml 10x TBE	**Stop solution:**	
36.6ml DDW	See 2.4.6	

2.9. DNA fingerprinting.

DNA fingerprinting was carried out in order to confirm that DNA from xenograft tissue corresponded to that of the primary tumour from which it had been established and to the normal tissue from that patient. This procedure was performed in a few selected cases where the results of the microsatellite analysis of the xenograft DNA were not consistent with the primary tumour. This applied to the xenograft 3xb which did not exhibit microsatellite instability, unlike the primary tumour it was established from and to one of the xenografts derived from tumour No 19 (19xa) which showed shifts at all four loci examined contrary to the primary tumour which microsatellites were stable. A few matched primary tumour/xenograft pairs were also analysed for comparison.

The probe 29C1 was chosen for DNA fingerprinting. It recognises a highly polymorphic region of human telomeric DNA located in the pairing regions of the short arms of the sex chromosomes (Cooke *et al.*, 1985).

2.9.1. Restriction endonuclease digestion of genomic DNA with EcoRI.

10μg of genomic DNA extracted from frozen tissue as described in section 2.3.1 was digested overnight at 37°C in a 50μl volume with 30U EcoRI and 1x enzyme buffer. The efficiency of the reaction was checked by running 5μl of the reaction mixture with 5μl bromophenol blue loading dye on 1% agarose gel with 5μl 10mg/ml ethidium bromide to confirm that digest was complete.

2.9.2. Electrophoresis and DNA transfer.

A 0.8% agarose horizontal gel 20x20cm was prepared. 2.4g electrophoresis-grade agarose was mixed with 300ml 1xTBE buffer and boiled until agarose dissolved. 5μl 10mg/ml ethidium bromide was added and the gel was left to cool to 55°C before pouring. 5μl bromophenol blue loading dye was added to each sample and the samples were loaded into the wells. 1μg of a DNA molecular weight marker 1kb ladder (Life Technologies Ltd) was loaded alongside the samples and electrophoresis was carried out overnight at 80mA in 1xTBE until the dye had migrated approximately 20cm. After the electrophoresis was completed the gel was photographed on a GEL DOC alongside a ruler for scale.

The gel was then prepared for blotting. It was shaken gently for 45 minutes in 500ml denaturing solution. It was very carefully transferred to the blotting apparatus which consisted of a 20x35cm of Whatman no.17 paper wick placed over a perspex plate. This was suspended over a tank containing denaturing solution. The wick was soaked in denaturing solution and carefully rolled flat before placing the gel on top. A sheet of Hybond N+ nylon membrane was cut to the size of the gel and one corner was cut off for orientation. It was soaked in DDW, then in denaturing solution, placed on top of the gel and rolled flat to remove bubbles. On top of this 2 sheets of Whatman no.17 chromatography paper cut to size of the gel were placed, followed by a pile of paper towels, a perspex plate and a 1kg weight. The edges of the gel were covered by clingfilm to prevent evaporation and aberrant buffer flow and the apparatus was left overnight for the DNA to transfer to the membrane.

10x EcoRI buffer:

500mM Tris HCl (pH8.0)

100mM $MgCl_2$

500mM NaCl

Denaturing solution:

0.5M NaOH

1.5M NaCl

2.9.3. Preparation of the probe.

2.9.3.1. Restriction endonuclease digestion of plasmid p29C1.

Pst1 restriction enzyme was used to cut out the insert from plasmid p29C1. The reaction mixture consisted of 20U enzyme, 4μg plasmid DNA and 1x Pst1 buffer made to the volume of 100μl with DDW. Digestion was carried out for 3 hours at 37°C.

2.9.3.2. DEAE membrane purification of the plasmid insert for the preparation of the probe.

The p29C1 plasmid insert was purified for use as a probe using DEAE (Schleicher and Schuell) membrane. Briefly, digested plasmid DNA was electrophoresed in 1% agarose gel at 100V in 1xTBE against 1kb ladder molecular weight marker for 1 hour. The 1.7kb insert was visualised briefly under UV light (to minimise DNA damage). A slit was cut in the gel, just in front of the required band, and a piece of DEAE membrane placed in the slit. The band was electrophoresed onto the membrane and then eluted from it by incubating at 65°C, for one hour, in a high salt buffer. The resulting DNA solution was extracted once with phenol:chloroform and then precipitated with ethanol. The DNA pellet was washed in 70% ethanol, air dried and then dissolved in sterile TE. Concentration was measured using CE 2020 spectrophotometer with readings of OD taken at 260nm. 50ng of insert was taken for labelling.

10xPst1 buffer:	**10xTBE:**	**High salt buffer:**
500mM Tris HCl (pH8.0)	See 2.4.2	50mM Tris
100mM $MgCl_2$		1mM EDTA
500mM NaCl		1M NaCl
		pH adjusted to 9.0

2.9.3.3. Labelling of the probe using random primers.

The random priming method was used for labelling probes with α^{32}PdCTP. The Prime-It RmT kit (Stratagene) was chosen for this purpose and it was used according to the protocol recommended by the manufacturers.

50ng of probe was added to a reaction tube containing random primers, nucleotides, buffer and co-factors for use with α^{32}PdCTP and denatured by boiling for 5 minutes. 50μCi of α^{32}PdCTP (ICN) and 12 units of DNA polymerase was added to this tube and the contents incubated at 37°C for 30 minutes. After half an hour the reaction was terminated by the addition of 2μl stop mix (0.5M EDTA, pH8.0). Unincorporated α^{32}PdCTP was removed with a Sephadex G-50 nick column (Pharmacia), used according to the manufacturers instructions. The column was rinsed with TE and equilibrated by allowing 3ml of TE to pass through the column. The labelled DNA probe was added along with 400μl TE and allowed to pass into the column bed. The probe was then eluted from the column by the addition of a further 400μl of TE and collected in an eppendorf. An approximate measure of isotope incorporation into the probe was obtained by comparing the Geiger counter reading of the tube relative to the column.

2.9.4 Hybridisation.

After completing Southern transfer overnight the nylon membrane was washed in neutralising buffer for 45 min. It was than placed onto a square nylon mesh, rolled, and placed into a Hybaid bottle with 20 ml hybridisation buffer to prehybridise in order to reduce non-specific background. The bottle was placed on a rotating wheel in a 65°C oven for 4 hours. After this time, probe prepared as above was denatured

by boiling for 3 minutes. The hybridisation buffer was removed from the bottle, the denatured probe added and the buffer returned to the bottle. The bottle was placed again on a rotating wheel in a 65°C oven for overnight hybridisation.

The next day the hybridisation buffer was poured out and the membrane was washed by adding 100ml 2xSSC prewarmed to 65°C and replacing the bottle in the oven at 65°C for 30 minutes. This procedure was repeated with 2xSSC/1% SDS and then 0.5xSSC/1% SDS. The membrane was then removed from the bottle, placed into 0.1xSSC at room temperature and soaked for 30 minutes. It was sealed in Clingfilm and placed in an X-ray cassette (Hypercassette, Amersham) containing two Hyperscreen (Amersham) intensifying screens and a sheet of Kodak X-OMAT film, and exposed at -70°C for 1-10 days. Then the film was developed using an automatic developing machine.

Neutralising buffer:

0.5M Tris pH 7.4

1.5M NaCl

20xSSC:

3M NaCl

0.3M Trisodium citrate

pH7

Hybridisation buffer:

5g dextran sulphate dissolved in 25ml DDW at 65°C for 30 minutes, followed by addition of:

15ml 20xSSC

2.5ml 20% SDS

heated to 65°C.

Immediately before use, 10mg salmon sperm DNA denatured by boiling for 5 minutes before adding to above buffer at 65°C.

pH to 7, DDW to 1l

2.10. DNA fingerprinting using microsatellite analysis.

This procedure was carried out in one case of the primary tumour No 3 and the xenograft 3xb, after the results of the DNA fingerprinting using 29C1 probe were inconclusive. A set of seven microsatellite markers was used. The analysis was performed by Mr Peter Han from the Police Forensic Science Laboratory and therefore will not be discussed here in details.

2.11. Investigating the origin of tumour cells in the xenograft 3xb by immunohistochemistry.

This was carried out on paraffin tissue sections by the research laboratory of the Department of Pathology, University of Edinburgh, following standard methodology for immunohistochemistry using antibodies against common leucocytic antigen CD45, B-cells antigen CD20, pan-T-cell marker CD3 and cytokeratin CAM 5.2.

Additionally, immunohistochemical detection of the Epstein-Barr virus was performed on paraffin tissue section from the 3xb xenograft by the research laboratory of the Western Infirmary Department of Pathology in Glasgow. A routine immunohistochemistry protocol was followed.

CHAPTER 3.

Spatial and temporal analysis of microsatellite instability in sporadic colorectal cancer.

3.1. Introduction.

One of the two major mechanisms of genomic instability identified to date in colorectal cancer leads to the development of a distinct phenotype characterised by an increased rate of frameshift and point mutations. This is manifested in a high rate of alterations in length of repetitive nucleotide sequences, known as microsatellite instability (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Eshleman *et al.*, 1995).

Microsatellites are normally stable repetitive genetic elements, where the repeating unit is one to six bases (Weber *et al.*, 1989). Because of their repetitive nature they are particularly prone to replication errors (Kunkel *et al.*, 1990), which in normal cells are swiftly corrected by the efficient mismatch repair system. Defects in MMR genes result in a failure to correct these errors, creating the replication error phenotype.

The RER+ phenotype is a characteristic of most tumours from patients with hereditary non-polyposis colorectal cancer, where it is a consequence of mutations in one of the DNA mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3* or *hMSH6*) (Peltomaki *et al.*, 1993; Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Papadopoulos *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996; Akiyama *et al.*, 1997; Miyaki *et al.*,1997). This phenotype is also detected in approximately 15-20% of sporadic colorectal cancers (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Wu *et al.*, 1994; Borresen *et al.*, 1995; Liu *et al.*, 1995; Bubb *et al.*, 1996; Eshleman and Markowitz, 1996; Konishi *et al.*, 1996). Inactivating mutations in MMR genes, however, appear to play a limited role in sporadic colorectal cancers with microsatellite instability. Recent studies suggest that inactivation of MMR system in sporadic tumours occurs through hypermethylation of CpG sites in the promoter region of *hMLH1* followed by loss of its expression (Cunningham *et al.*, 1998; Herman *et al*, 1998; Deng *et al.*, 1999; Maekawa *et al.*, 1999; Wheeler *et al.*, 1999).

There are still many unresolved issues regarding the diagnosis of a tumour as RER+. They include the degree to which the microsatellite sequence has shifted compared to the normal allele (Thibodeau *et al.*, 1993), the number of shifted microsatellite loci sufficient to diagnose the tumour as RER+ (Aaltonen *et al.*, 1993) and the optimal set of informative loci (Liu *et al.*, 1995).

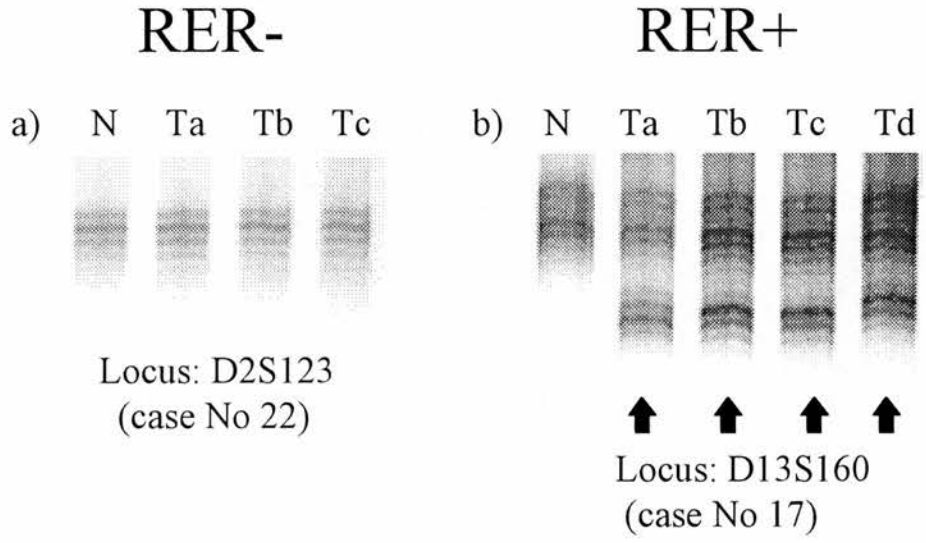
The aim of this study was to determine the RER phenotype of 22 primary sporadic colorectal carcinomas which were randomly chosen for this project. The analysis of samples taken from multiple sites from each tumour and the corresponding xenografts was designed to gain more information about the genetic intratumoral heterogeneity and the temporal evolution of microsatellite instability in sporadic colorectal cancer.

3.2. Materials and methods.

For the purpose of this study two dinucleotide repeat sequences D2S123 and D13S160 (Gyapay *et al.*, 1994), the poly (A) tract *BAT-26* (Hoang *et al.*, 1997) and the $(A)_{10}$ repeat in exon 3 of *TGFβ RII* were chosen to determine the RER phenotype of the primary tumours and the xenografts. Details of the methods can be found in section 2.4. The samples were classified as RER+ if they displayed band shifts at two or more loci and the primary tumours were considered RER+ if any of the multiple samples analysed met these criteria (see Figure 5).

Clinicopathological features of these tumours (ie. tumour location, Dukes' stage, histological features) were assessed based on the histopathology reports. Unfortunately the patients' survival data were not available since the tumours were collected at the time of this study.

Figure 5. *Analysis of microsatellite instability using autoradiography with ^{33}P-labelled PCR amplified sequences after electrophoresis on denaturing acrylamide gel. Tumours were classified as RER+ if they displayed microsatellite instability (generation of shorter or longer fragments in the tumour DNA compared to normal DNA) at two or more loci at any of the multiple sites examined.*



N, normal control
Ta, Tb, Tc, Td, multiple samples collected from one primary tumour

a) An example of RER- tumour.
b) An example of RER+ tumour. In this case shifts in allele size at locus D13S160 are present at all sites examined.

3.3. Results.

3.3.1. Analysis of microsatellite instability in primary tumours.

Five out of 22 primary colorectal cancers (22%) included in this study were classified as RER+. None of these patients met the Amsterdam Criteria (Vasen *et al.*, 1991) for diagnosing hereditary non-polyposis colorectal cancer (the presence of three or more family members with colorectal cancer in at least two successive generations, with at least one affected member having been diagnosed at less than 50 years of age). These tumours therefore represent RER+ cases of sporadic colorectal cancer.

The clinicopathological features of RER+ tumours are summarised in Table 5.

Table 5. *Clinicopathological features of RER+ colorectal cancers.*

Sample ID:	Patient's age	Dukes' stage	Tumour site & side	Differen-tiation	Mucinous
3	64	A	asc.col./right	moderate	-
12	60	C	asc.col./right	poor	-/+ (signet ring cells)
17	76	C	caecum/right	poor	-/+
18	77	B	asc.col./right	poor	-
20	70	C	asc.col./right	poor	+

asc.col, ascending colon
-, no evidence of mucin production
-/+, mucinous pattern present, but comprises less than 60% of the tumour and therefore the tumour can not be classified as a mucinous adenocarcinoma
+, mucinous pattern comprises more than 60% of the tumour

The age of patients with RER+ colorectal cancer ranged from 60 to 77 in this study. Three of the five tumours were classified as Dukes' stage C, one Dukes' stage B and one Dukes' stage A. All of the RER+ tumours were right sided cancers (located in the proximal part of the large bowel up to and including splenic flexure), four being located in the ascending colon and one in the caecum. Four of these adenocarcinomas showed poor differentiation and three of these showed evidence of mucin production (see Table 5). Only one was classified as moderately differentiated adenocarcinoma.

The association of RER+ phenotype with p53 status, tumour DNA content and chromosomal abnormalities is discussed later (see Chapter 4 and 5).

3.3.2. Analysis of microsatellite instability at diferrent tumour sites.

Analysis of all four microsatellite loci at different tumour sites revealed the presence of substantial genetic heterogeneity in all of the tumours. In three of the five tumours the number of microsatellite loci affected and the allele size of affected loci differed between different sites within the tumour. In the two remaining cancers, only allele length differed between sites but the same loci were affected within these tumours (see Figure 6 and Table 6).

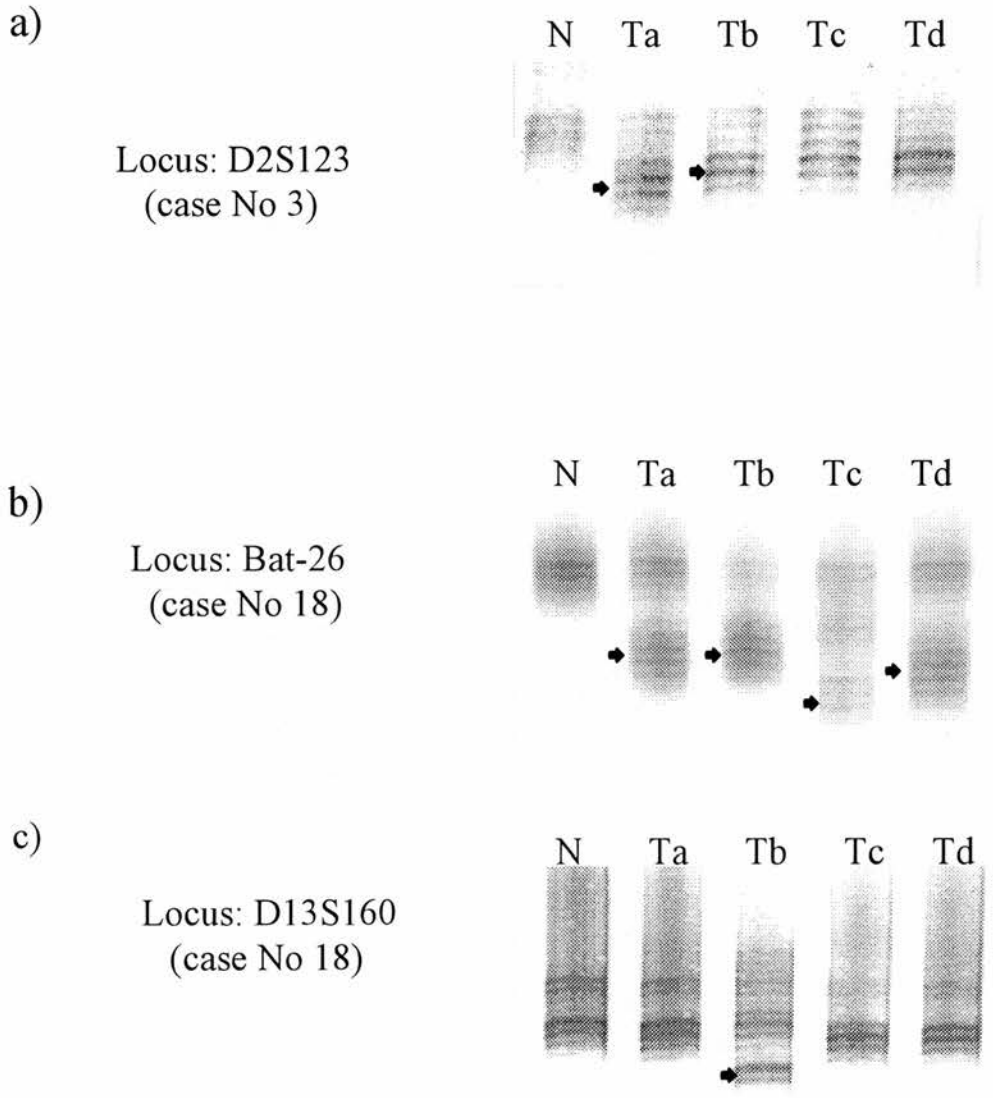
In case No 18, two of the four samples collected from the tumour (18a and 18d) did not meet the criteria employed to establish the RER+ phenotype and they showed a shift in allele size at only one locus (see Table 6). However, the tumour was classified as RER+ since two other samples showed shifts at multiple loci (18b and 18c).

Shifts in allele size were not detected at any of the four microsatellite loci examined in any of the multiple samples analysed from RER- primary tumours.

Table 6. *Presence of shifts in allele size at four microsatellite loci examined at different tumour sites in RER+ cancers.*

Sample	D2S123	D13S160	BAT-26	TGFβ RII	RER status
3a	shift	-	shift	shift	+
3b	shift	-	shift	shift	+
3c	shift	-	shift	shift	+
3d	shift	-	shift	shift	+
12a	shift	shift	shift	shift	+
12b	shift	shift	shift	shift	+
12c	shift	shift	shift	shift	+
12d	shift	shift	shift	shift	+
17a	-	shift	shift	shift	+
17b	-	shift	shift	shift	+
17c	shift	shift	shift	shift	+
17d	shift	shift	shift	shift	+
18a	-	-	shift	-	-
18b	-	shift	shift	-	+
18c	shift	-	shift	-	+
18d	-	-	shift	-	-
20a	-	shift	shift	shift	+
20b	-	-	shift	shift	+
20c	-	shift	shift	shift	+
20d	-	-	shift	shift	+

Figure 6. *Analysis of microsatellite instability using autoradiography with ^{33}P-labelled PCR amplified sequences after electrophoresis on denaturing acrylamide gel. This figure shows heterogeneity of microsatellites between different tumour sites.*



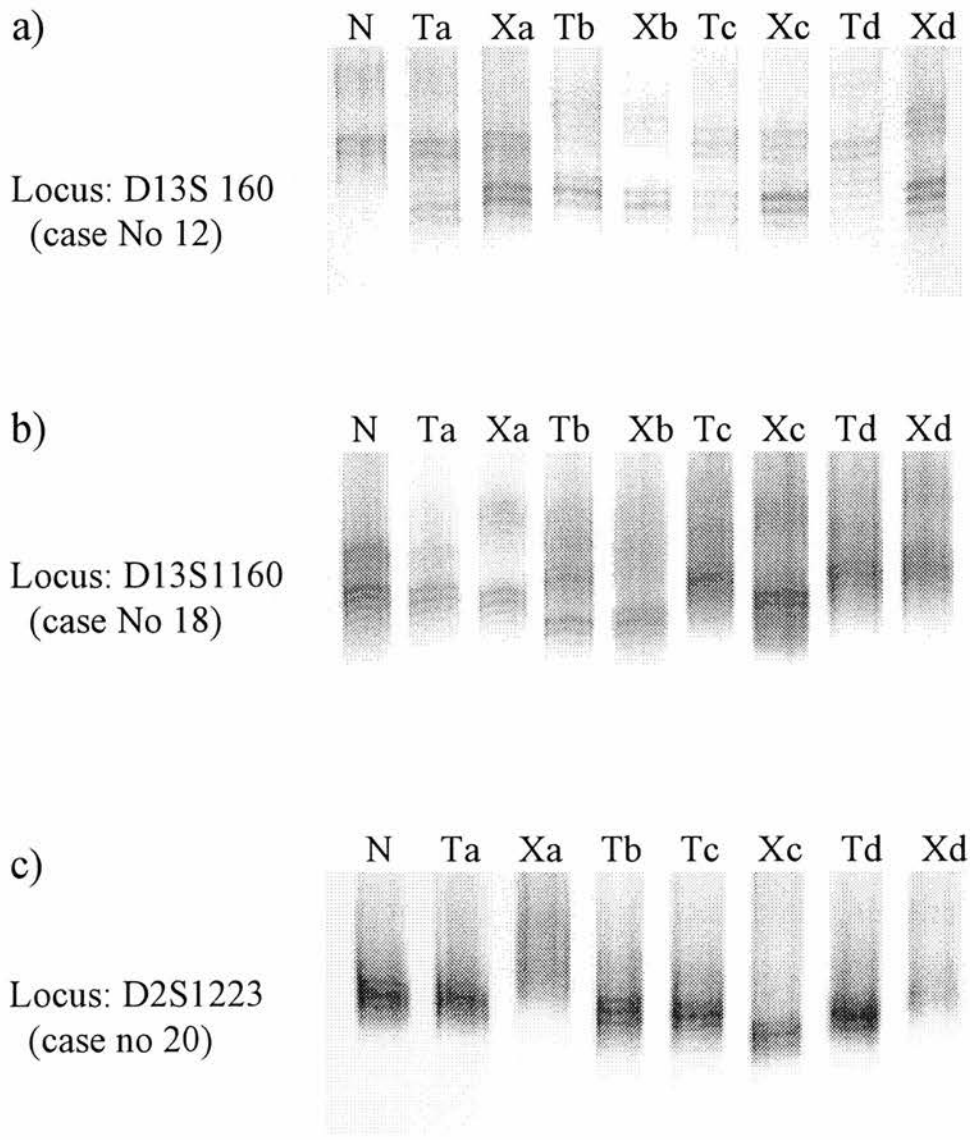
N, normal control
Ta, Tb, Tc, Td, multiple samples collected from one primary tumour

a) Tumour showing microsatellite instability detected at all tumour sites examined; however, the allele length differs at the site Ta not only compared to normal N, but also to allele sizes at the other sites.
b) Tumour showing microsatellite instability with different allele length at each of the examined sites.
c) Tumour showing shifts in allele size only at one of the four sites examined.

3.3.3. Temporal evolution of microsatellite sequences in sporadic colorectal cancer with underlying microsatellite instability, as reflected by the analysis of xenografts established in SCID mice.

Colorectal cancer xenografts were successfully established from samples collected from multiple sites from three RER+ tumours (see section 2.2 for details). Analysis of the selected microsatellite loci in DNA from the normal tissue, primary tumour tissue and corresponding xenograft tissue revealed additional changes in both numbers of altered microsatellite loci and size of altered alleles in xenografts compared with primary tumours and normal tissue (see Figure 7). The RER+ phenotype was always preserved in colorectal cancer xenografts established from tumours with MIN regardless of sample site. In one case (3xb, see Appendix 1b) a xenograft established from a RER+ tumour appeared to have lost its RER+ phenotype but further investigation allowed the identification of this xenograft as a B-cell lymphoma and not an adenocarcinoma. Therefore, this interesting case has been excluded from this study and is discussed in detail in Chapter 6. None of the xenografts established from RER- tumours acquired a RER+ phenotype during passage in SCID mice, which on average lasted 12 weeks. One of the three xenografts established from RER- tumour No 22 (22xb, see Appendix 1) acquired a shift in one of the four loci that was not present in the sample from the primary tumour but this did not satisfy the RER+ criteria. In addition xenograft 19xa unexpectedly showed allele size shifts at all four loci, unlike the sample from the primary tumour (19a), where no shifts were detected. However, DNA fingerprinting of this xenograft failed to match this sample to the normal tissue of the patient from whose tumour the xenograft was established. It was therefore excluded from this study but the case is described in detail in Chapter 6.

Figure 7. *Analysis of microsatellite instability using autoradiography with ^{33}P-labelled PCR amplified sequences after electrophoresis on denaturing acrylamide gel. Three examples of changes in the number of loci and size of alleles affected in xenografts compared to primary tumour and normal tissue.*



N, normal control
Ta, Tb, Tc, Td, multiple samples collected from one primary tumour
Xa, Xb, Xc, Xd, xenografts established from samples Ta,Tb,Tc and Td collected from the primary tumour.

The results of the microsatellite analysis in primary tumours sampled at multiple sites and in corresponding xenografts are summarised in Table 7.

Table 7. *Presence and distribution of shifts in allele size at the examined loci in RER+ tumours sampled at multiple sites and in corresponding xenografts.*

Locus:	D2S123		D13S160		BAT-26		TGF-beta RII	
Sample ID:	p	x	p	x	p	x	p	x
3a	⇨	**na**	o	**na**	⇨	**na**	⇨	**na**
3b	⇨	**na**	o	**na**	⇨	**na**	⇨	**na**
3c	⇨	**na**	o	**na**	⇨	**na**	⇨	**na**
3d	⇨	**na**	o	**na**	⇨	**na**	⇨	**na**
12a	⇨ ★	⇨ ★	⇨	⇨	⇨	⇨ ★	⇨	⇨ ★
12b	⇨ ★	⇨ ★	⇨	⇨ ★	⇨	⇨ ★	⇨	⇨ ★
12c	⇨ ★	⇨ ★	⇨	⇨	⇨	⇨ ★	⇨	⇨ ★
12d	⇨ ★	⇨ ★	⇨	➡ ★	⇨	⇨ ★	⇨	⇨ ★
17a	o	**na**	⇨	**na**	⇨	**na**	⇨	**na**
17b	o	**na**	⇨	**na**	⇨	**na**	⇨	**na**
17c	⇨	**na**	⇨	**na**	⇨	**na**	⇨	**na**
17d	⇨	**na**	⇨	**na**	⇨	**na**	⇨	**na**
18a	o	⇨	o	⇨	⇨	⇨ ★	⇨	⇨ ★
18b	o	⇨	⇨	⇨ ★	⇨	⇨ ★	⇨	⇨ ★
18c	⇨	⇨	o	⇨ ★	➡	➡ ★	⇨	⇨ ★
18d	o	⇨	o	⇨	⇨	➡ ★	⇨	⇨ ★
20a	o	⇨ ★	⇨	⇨	⇨	⇨ ★	⇨	⇨
20b	o	**na**	o	**na**	⇨	**na**	⇨	**na**
20c	o	⇨ ★	⇨	⇨ ★	⇨	⇨ ★	⇨	⇨
20d	o	⇨ ★	o	o	⇨	⇨ ★	⇨	⇨

o indicates no shift in allele size from normal
⇨ indicates a shift in allele size from normal, different shades (grey scale) indicate differences in allele size
na not available
p primary tumour
x xenograft
★ indicates complete loss of normal allele in the xenograft

3.4. Discussion.

This study of 22 sporadic colorectal cancers identified 5 tumours (22%) exhibiting microsatellite instability. These results are in concordance with the previously published data, stating the prevalence of the RER+ phenotype in sporadic colorectal cancer to be 15-20% (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Wu *et al.*, 1994; Borresen *et al.*, 1995; Liu *et al.*, 1995; Bubb *et al.*, 1996; Eshleman and Markowitz, 1996; Konishi *et al.*, 1996). Clinicopathological features of RER+ colorectal cancers have been described in detail (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Kim *et al.*, 1994; Bubb *et al.*, 1996; Senba *et al*, 1998) and our data are in concordance with the published results (Table 5). This includes predominant location in the proximal colon and characteristic histopathological features (poor differentiation and evidence of mucin production).

The results of the analysis of microsatellite instability at different tumour sites indicate that despite the presence of substantial intratumoral heterogeneity, in the majority of cases the RER+ phenotype could be readily identified by analysing a single tumour sample. Only in one of the five cases analysed was this not always possible. In this instance two out of four samples collected from the tumour (18a and 18d) showed allele size shifts at one locus only, which on their own would not have satisfied the criteria employed in this study for defining the RER+ phenotype. However, there is still a disagreement concerning the minimal number of microsatellite loci that should be studied and what proportion of loci displaying instability should result in the classification of a tumour as RER+. A number of authors have classified tumours as RER+ when as few as one of two loci appeared unstable (Chong *et al.*, 1994). Our data show that even when more stringent criteria are employed, single sample analysis is usually sufficient for detecting RER+ phenotype.

The analysis of four microsatellite loci at different sites within RER+ tumours and in the corresponding xenografts confirmed the presence of substantial genetic intratumoural heterogeneity regarding the number and size of alleles affected and revealed the temporal evolution of these changes in the colorectal cancer xenografts. These observations remain in concordance with the hypothesis that MIN is a consequence of an underlying mechanism of genetic instability which continuously

targets repetitive DNA motifs and leads to increased numbers of frameshift mutations. Our data suggest that the defect in the mismatch repair system responsible for these mutations either occurs early in colorectal carcinogenesis, or confers substantial growth advantage on the tumour cells, thus permitting massive clonal expansion. High homogeneity of the resulting RER+ phenotype, which can be readily detected at different sites within a tumour supports this hypothesis. It also confirms that once this defect is acquired, the RER+ phenotype that subsequently develops is preserved in colorectal cancer xenografts. Further, the data emphasise the multiplicity of clonal divergence due to repeated episodes of mismatch at microsatellite sites in these tumours.

Previous studies demonstrated the maintenance of the RER+ phenotype *in vitro* and *in vivo* (Shibata *et al.*, 1994; Ottini *et al.*, 1997; Curtis, 1998) and our results are in concordance with this data. They additionally prove the colorectal cancer xenografts to be highly representative of the primary tumour, as far as RER status is concerned, regardless of genetic intratumoral heterogeneity. Although it is possibile that additional genes are targeted in xenografts compared to the primary tumour, colorectal cancer xenografts still represent an extremely valuable tool in studies on colorectal cancer genetics and treatment response.

3.5. Summary.

In summary, this study has confirmed that a single sample analysis is sufficient for determining RER+ phenotype in sporadic colorectal cancer. It has also shown that the RER+ phenotype is characteristic of a proportion of sporadic colorectal cancers where it is preserved in RER+ tumour cells during passage in SCID mice. It is likely to have occurred early during tumour development. This is supported by its homogenous distribution within RER+ tumours and by the fact that none of the RER- colorectal cancers acquired this phenotype during passage in SCID mice. The multiplicity of clonal divergence in RER+ colorectal tumours detected in this study further supports the high mutation incidence in these tumours. This study has determined that colorectal cancer xenografts are representative of the tumour of origin with regard to RER status.

CHAPTER 4.

Spatial and temporal analysis of chromosomal instability in sporadic colorectal cancer.

4.1. Introduction.

A distinct phenotype identified in colorectal cancer is characterised by an increased number of chromosomal abnormalities. This includes both gain or loss of individual chromosomes and structural alterations within chromosomes consisting of translocations, chromosome deletions, inversions and gene amplifications. The former can easily occur through a non-disjunction event, while the latter are believed to proceed via chromosomal breaks (Gerdes *et al.*, 1995; Ried *et al.*, 1996; Lengauer *et al.*, 1997b; Cahill *et al.*, 1998; Eshleman *et al.*, 1998a).

Abnormal chromosome copy number (aneuploidy) is nearly ubiquitous in cancer (Mertens *et al.*, 1997; Mitleman *et al.*, 1997). It could therefore be argued that it results simply from the abnormal structure and growth properties of cancer cells. There is however increasing evidence for its association with underlying chromosomal instability (Lengauer *et al.*, 1997b; Cahill *et al.*, 1998). CIN has been suggested as an alternative pathway in colorectal carcinogenesis where the defect in the mechanism controlling chromosome segregation and the resultant chromosomal instability drives the tumorigenic process, just as defective mismatch repair drives neoplasia in MIN tumours (Lengauer *et al.*, 1997b). The potential molecular mechanisms leading to the development of CIN phenotype have been reviewed in Chapter 1 (see 1.2.6.3).

Different mechanisms are thought to be responsible for structural chromosomal aberrations. Complex translocations are frequently observed in many solid tumours. Although the molecular basis for the translocations in cancers is not known, it has been suggested that they arise in cells that enter mitosis before recombination-promoting double-strand breaks are repaired (Elledge, 1996; Paulovich *et al.*, 1997). Defects in genes such as *ATM*, *ATR*, *BRCA1*, *BRCA2*, *p53* and other genes involved in double-strand breaks repair have been suggested to be involved in this process

(Lengauer *et al.*, 1998). Amplifications of oncogenes occur in a subset of late-stage cancers of many organs, and amplification of genes involved in metabolism or inactivation of drugs represents a common way for cultured cells to acquire resistance to chemotherapeutic agents. Although amplifications can significantly affect tumour biology, they affect only a single or a few genes in each cell and, in general, occur late in tumorigenesis. The mechanisms through which amplifications are generated are largely unknown (Lengauer *et al.*, 1998).

A number of studies have revealed a strikingly lower incidence of chromosomal alterations in the RER+ colon cancers in comparison with RER- tumours. This supports the hypothesis that the pathway of RER+ colon carcinogenesis is fundamentally different from that of RER- and strongly links the RER- phenotype with CIN (Bardi *et al.*, 1995; Schlegel *et al.*, 1995; Lengauer *et al.*, 1997b; Eshleman *et al.*, 1998a).

The aims of this study were to determine the following:

1. The prevalence of CIN in sporadic colorectal cancer and its relationship to RER status.
2. Whether, as previous studies suggested, CIN is always detected in RER- colorectal tumours and therefore can be considered an underlying mechanism of tumorigenesis in RER- cancers.
3. Patterns of chromosomal abnormalities in RER- and RER+ sporadic colorectal cancers.
4. Chromosomal change through time in RER- and RER+ cancers.
5. Whether colorectal cancer xenografts are representative of the tumours of origin with regard to chromosomal abnormalities.

These aims were achieved by establishing the chromosome copy number changes, using Comparative Genomic Hybridisation at multiple sites in primary tumours (see 2.5 for materials and methods). This allowed the investigation of the clonal evolution of chromosomal changes in sporadic colorectal cancer and identification of chromosomal abnormalities specifically selected for during tumour development. In addition, the extent of chromosomal change through time was assessed by the

examination by CGH of multiple colorectal cancer xenografts established in SCID mice.

4.2. Materials and methods.

Chromosomal copy number changes detected by Comparative Genomic Hybridisation were examined in the same 22 primary colorectal cancers, sampled at multiple sites, in which RER status had previously been determined (see Chapter 3).

CGH detects changes in chromosome copy number (whole or partial) through competitive hybridisation of DNA derived from normal and tumour tissue onto a normal human chromosome metaphase spread (Kallioniemi *et al.*, 1992). Normal and test DNA are differentiated by fluorescent coloured labels (red and green respectively) and a fluorescent ratio is assessed along the length of each chromosome. Regions of amplification or deletion are detected as a change in the ratio of the two colours fluorescence. Unlike a conventional amplification/deletion analysis, which is limited to a single locus, CGH provides information about the whole genome at once and therefore is a considerably more powerful tool. However, unlike conventional cytogenetic analysis it cannot detect balanced chromosomal changes such as translocations or pure polyploidisation. Similarly, it gives no indication of the character of the structural rearrangements that might be responsible for the deletions and amplifications that it does identify.

Chromosomal abnormalities detected by CGH were found to consist mostly of deletions or amplification of either whole chromosomes or entire chromosome arms. Very occasionally changes that involved interstitial breakpoints and led to the loss of an arm fragment simultaneous with gain of the remaining part of the chromosome arm were observed and these changes were scored independently.

From these raw data several different indices were extracted. First, the frequency with which individual chromosome arms are lost or gained in the RER- and RER+ tumours was calculated. In order to determine the most frequent chromosomal changes in colorectal cancers, the prevalence of a particular chromosomal change in RER- and RER+ tumours was scored. This was expressed as the percentage of all RER- or RER+ colorectal cancers in which particular chromosomal abnormality was observed,

regardless of whether it was detected only at one or more of the multiple sites examined in each tumour.

The CGH analysis of the multiple sites within colorectal cancers allowed identification of chromosomal changes that either occurred early in the tumour development or were selected for during tumour progression. These were changes which were present at the majority of the examined sites within tumours, regardless of the proportion of colorectal cancers in which they were identified (see Figure 10 and 13, Table 8 and 9).

A chromosomal instability index (CIN index) was calculated for each tumour in order to assess the level of underlying chromosomal instability in RER+ and RER- tumours. Two factors that reflect chromosomal instability were assessed; the mean number of chromosomal gains and losses within a tumour, and heterogeneity of the presence of particular chromosomal changes within a tumour. The CIN index was expressed as a sum of these two scores (see section 4.3.5). This index was also calculated in colorectal cancer xenografts established from RER+ and RER- tumours, when these could be established successfully from more than one site. This provided additional information about the progress of chromosomal changes in time in both cancer groups.

Finally these indices were compared with data on nuclear DNA content, obtained by Flow Cytometry of propidium iodide stained nuclei prepared by the method of Vindelov *et al.* (1983) as described in section 2.6.

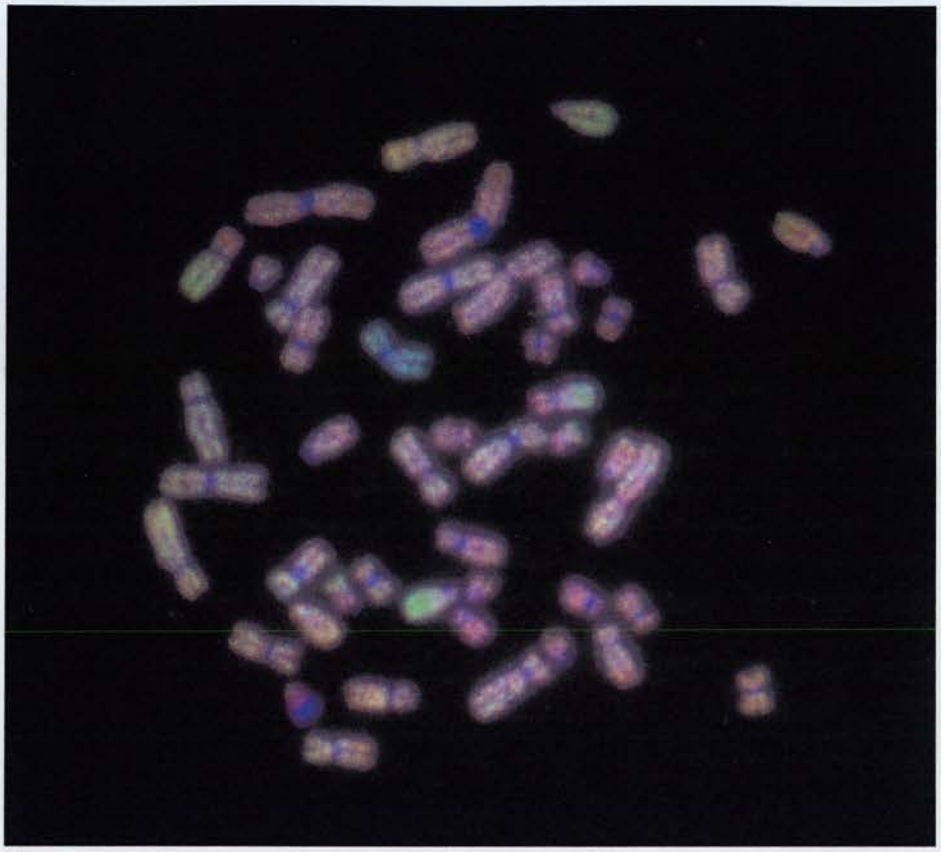
4.3.1. Several chromosomes are consistently abnormal in RER- cancers.

A characteristic pattern of chromosomal abnormalities appears to be present in the majority of RER- colorectal cancers. The commonest chromosomal abnormality identified by CGH in RER- cancer was gain of the long arm of chromosome 20 observed in 88% of tumours (Figure 10). Next in frequency came 18q- (76%), 13q+ (64%), 8p- (59%) often in association with 8q+ (53%) and 1p-, 18p-, 7p+, 4p- (each present in 53% of RER- tumours). In 2 cases (12%) all of these chromosomal abnormalities were present in one tumour. More frequently it was a combination of the above changes. The combination of at least 8 out of 9 of these chromosomal changes was present in 4 tumours (24%). At least 7 of these changes were present in 8 tumours (47%), 9 tumours (53%) showed the presence of at least 6 characteristic chromosomal abnormalities and at least 5 of these changes were detected in 10 tumours (59%). All of the 17 RER- cancers showed the presence of at least one of the common chromosomal abnormalities.

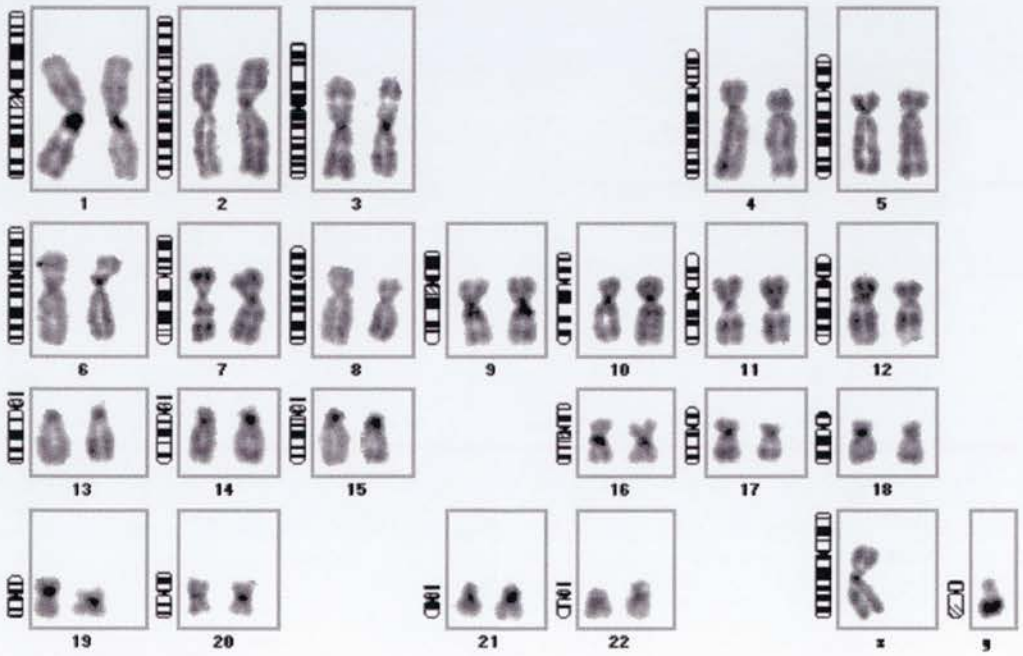
Moreover, most of these changes (20q+, 18q-, 13q+, 8p-, 1p- and 8q+) when observed in any tumour were found in 50% or over of the sampled sites suggesting they may be acquired in time close to the establishment of the founder clone of the carcinoma. In contrast, whilst every chromosomal arm showed some abnormality in at least one tumour, most of the less frequent alterations also occurred in only a minimal subset of the sites sampled within those tumours in which they were observed at all (Figure 10).

Figure 8. *Example of CGH of a RER- primary tumour.*

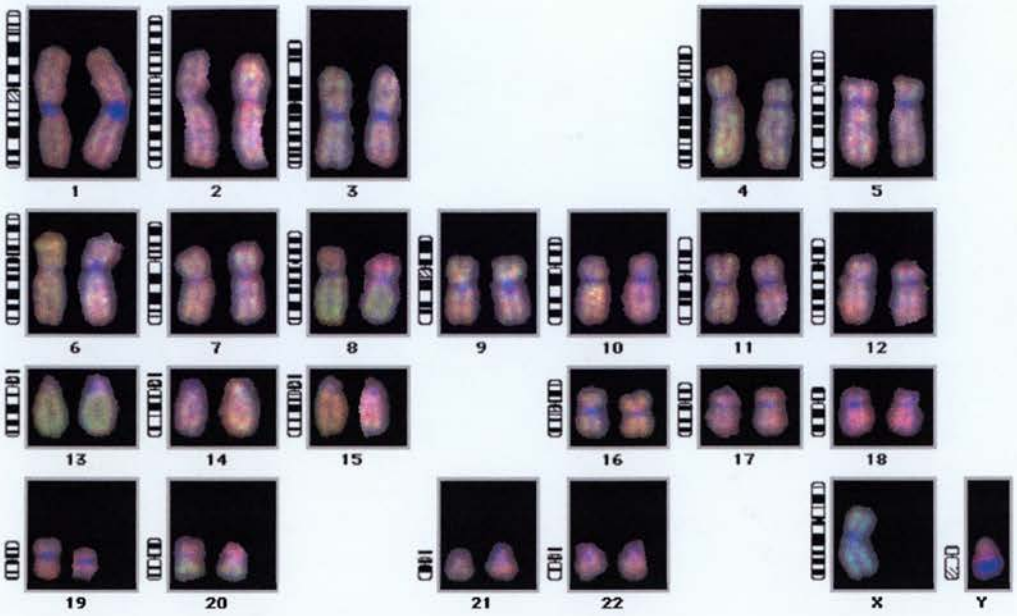
a) Metaphase image (case No 22c).



b) Normal human karyotype with DAPI banding.



c) Metaphase after karyotyping.



d) Average green/red ratio profile (blue line) calculated from 6 metaphases. Cut-off points of 0.875 (red line) and 1.125 (green line) were chosen for scoring of chromosome copy number. Thick lines next to chromosome ideograms indicate loss (red) or gain (green) of chromosome material. This profile shows numerous chromosomal abnormalities detected: 1 del, 4q+, 8p-, 8q+, 12q-, 13q+, 17p-, 18 del, 19q-, 20p-,20q+,22q-.

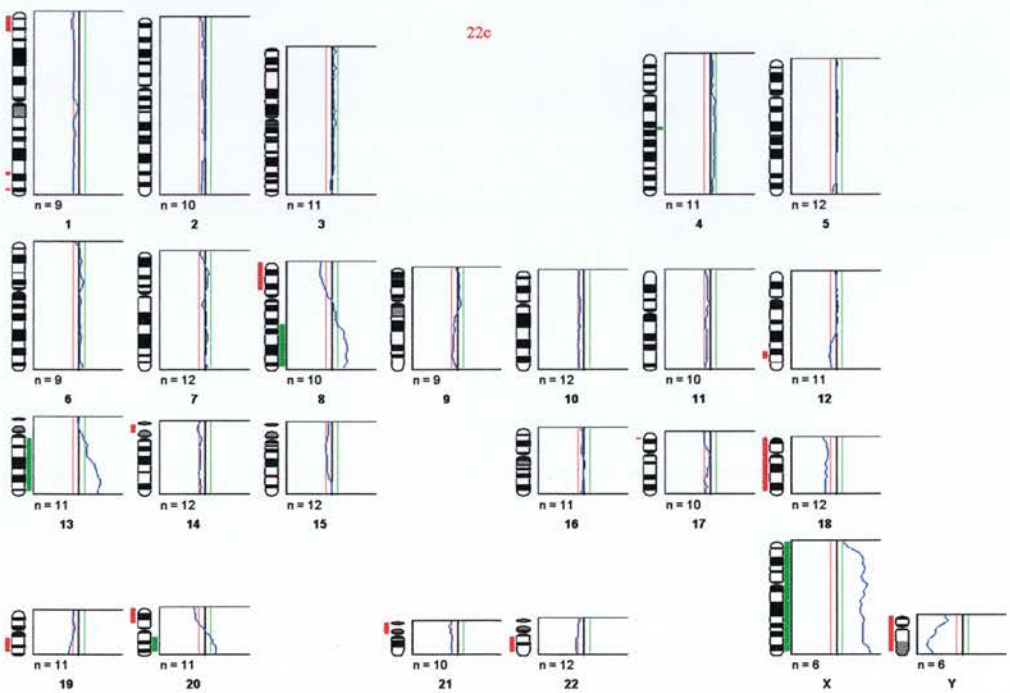


Figure 9. *Chromosome copy number changes as detected by CGH in RER- colorectal cancers sampled at multiple sites.*

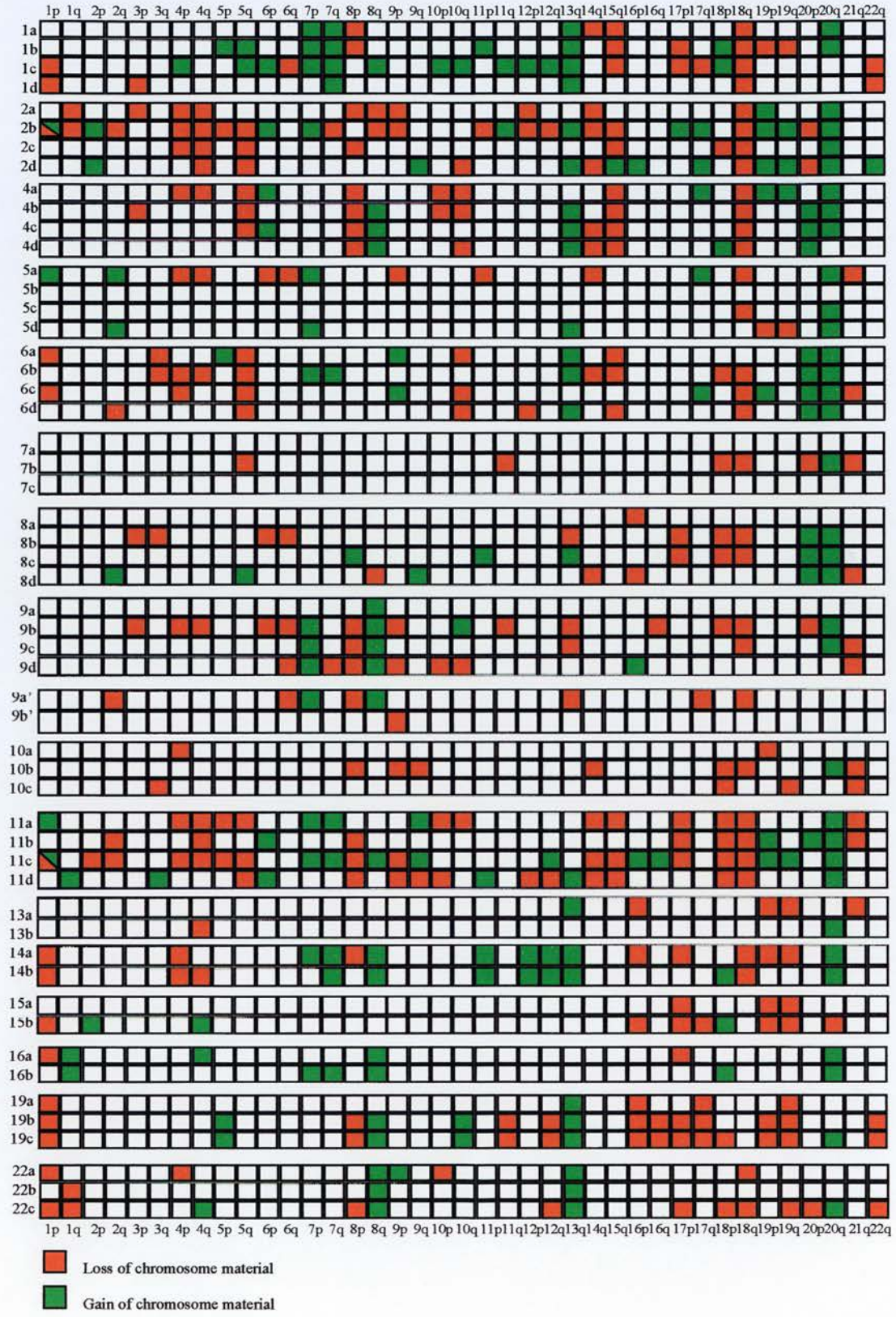
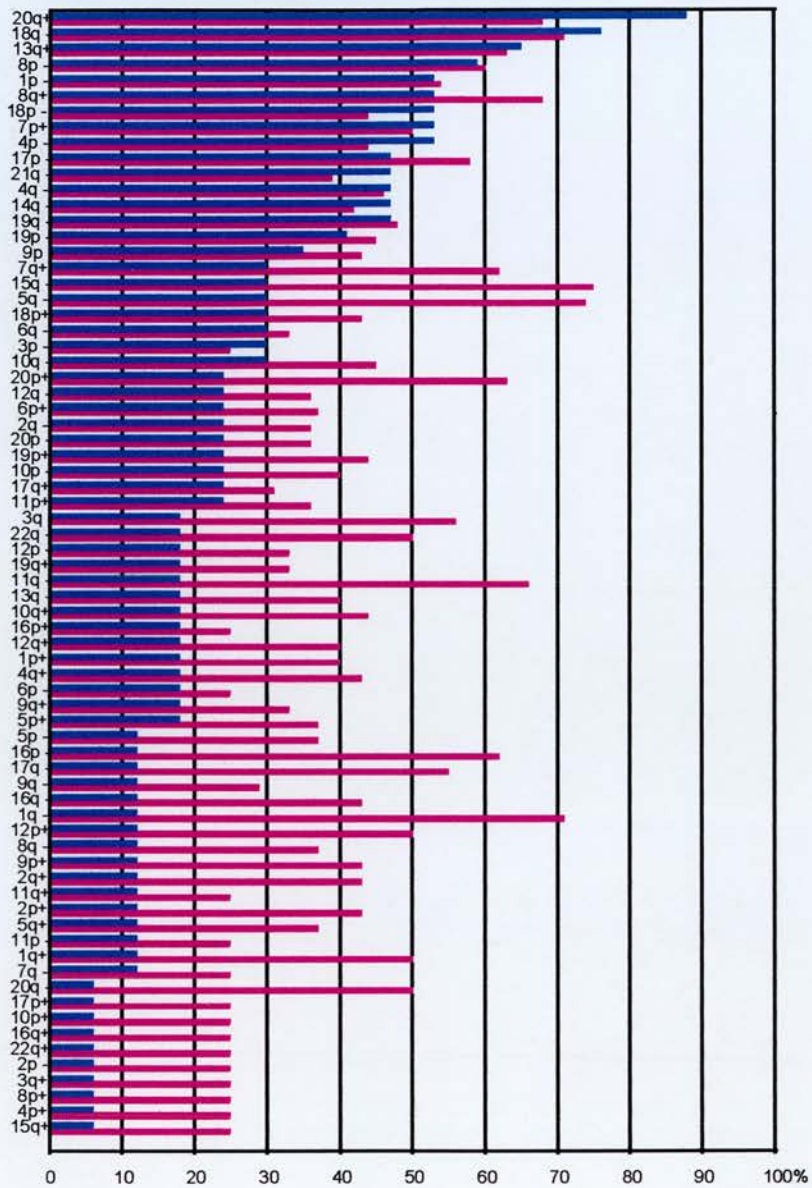


Figure 10. *Frequency of occurrence of chromosomal abnormalities and their prevalence at different tumour sites in RER- colorectal cancers.*



Frequency of a particular chromosomal change (gain + or loss - of chromosome material) in RER- colorectal cancers. This number was calculated as a percentage of all RER- colorectal tumours examined in which the particular abnormality was observed at any of the multiple sites examined.

Prevalence of a particular chromosomal change within RER- primary tumours. This number was calculated as the ratio between the number of sites at which a particular chromosomal abnormality was detected and the total number of sites examined averaged over all the tumours where a particular abnormality occured

Table 8. *Chromosomal abnormalities most frequently detected in RER- colorectal cancers.*

Chromosomal changes present in >50% RER- colorectal cancer cases examined	20q+, 18q-, 13q+, 8p-, 1p-, 8q+, 18p-, 7p+, 4p-
Chromosomal changes present at >50% of different sites examined within a tumour in RER- colorectal cancer	15q-, 5q-, 18q-, 1q-, 20q+, 8q+, 11q-, 13q+, 20p+, 7q+, 16p-, 8p-, 17p-, 3q-, 17q-,1p-
Chromosomal changes present in >50% of tumours at >50% of sites within a tumour in RER- colorectal cancer	**20q+, 18q-, 13q+, 8p-, 1p-, 8q+**

Chromosomal changes present in >50% of tumours at >50% of sites within a tumour are indicated in colour: loss of chromosome arm - red, gain of chromosome arm - green.

4.3.2. Chromosomal abnormalities in RER+ colorectal cancers.

An analysis of the most frequent chromosomal changes and changes selected for in RER+ colorectal cancers was carried out. This group of tumours, as expected, was substantially less numerous than the group of RER- cancers, rendering this analysis somewhat difficult (see Figure 13 for details). Nevertheless the analysis of chromosomal gains and losses detected by CGH identified a different pattern of chromosomal abnormalities in RER+ tumours.

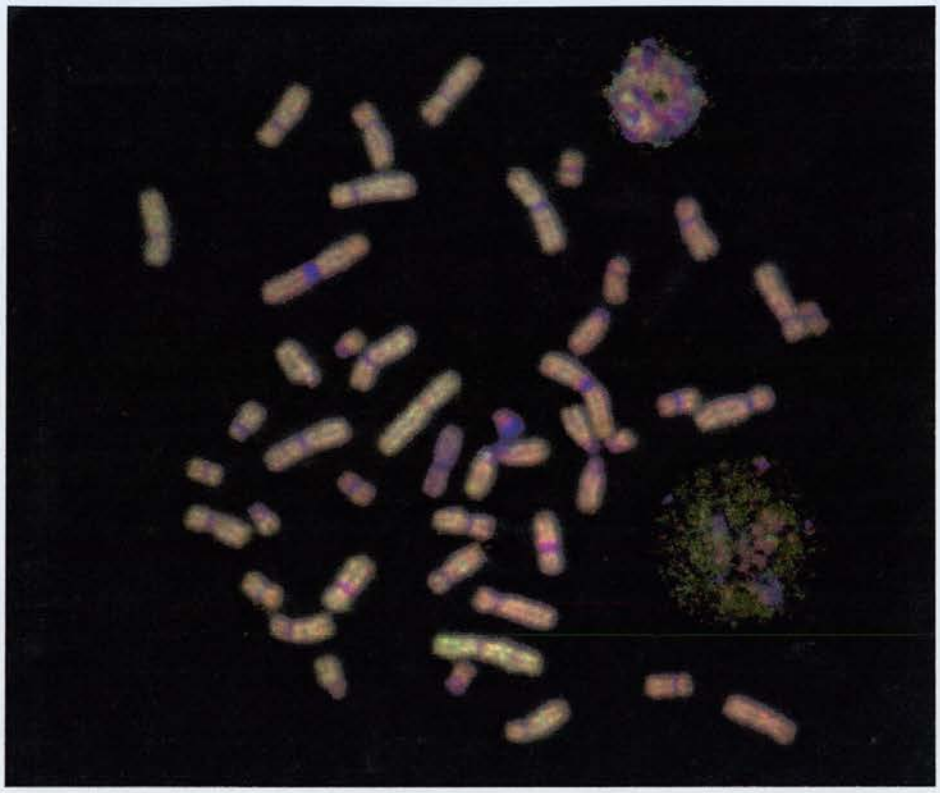
First of all, chromosomal abnormalities were much less frequent in RER+ cancers compared to RER- tumours. Detailed analysis of these differences was carried out and the results are described later in this chapter (see section 4.3.3).

A different subset of chromosomal changes appeared most frequently in RER+ cancers (Figure 13). These were loss of the short arm of chromosome 1 and the whole of chromosome 19. These results are summarised in Table 9.

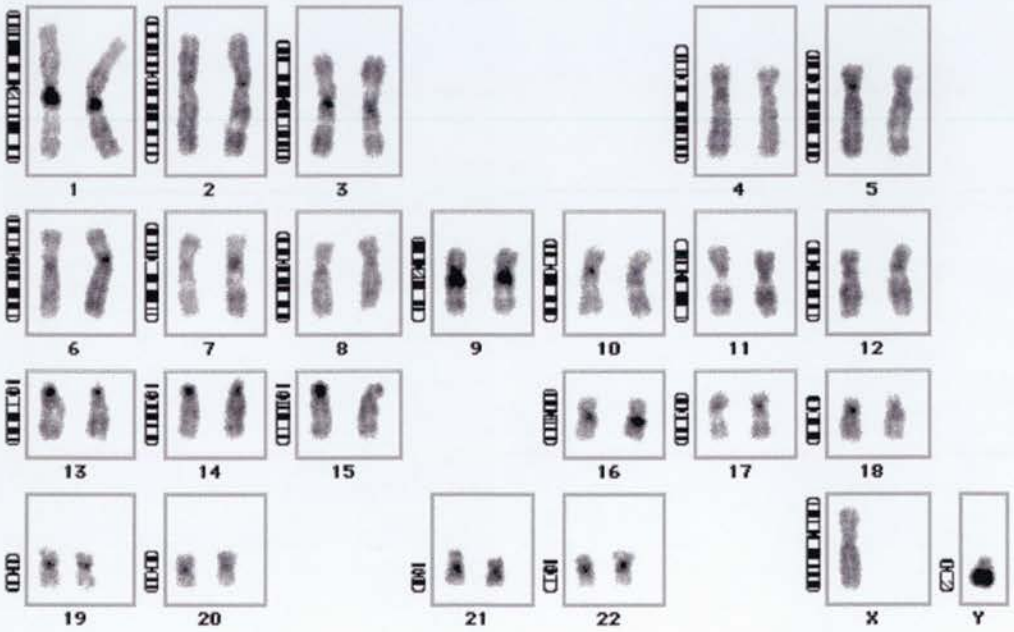
Figure 11 shows an example of CGH analysis of one sample collected from a RER+ tumour. Figure 12 presents the results of CGH analysis of primary RER+ colorectal cancers sampled at multiple sites.

Figure 11. *Example of CGH of a RER+ primary tumour.*

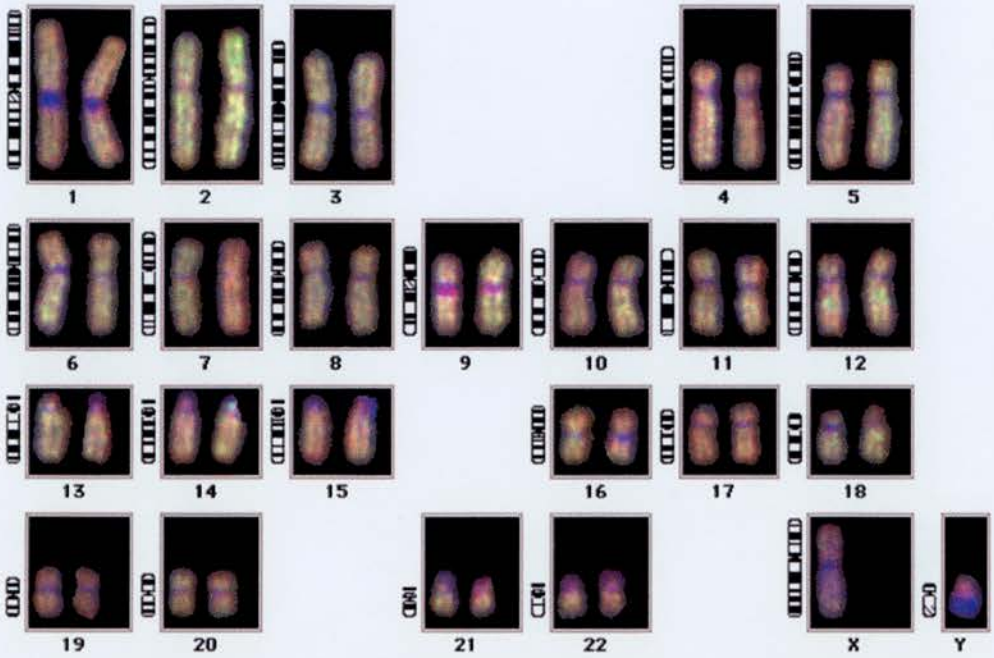
a) Metaphase image (case No 17b).



b) Normal human karyotype with DAPI banding.



c) Metaphase after karyotyping.



d) Average green/red ratio profile (blue line) calculated from 6 metaphases. Cut-off points of 0.875 (red line) and 1.125 (green line) were chosen for scoring of chromosome copy number. Thick lines next to chromosome ideograms indicate loss (red) or gain (green) of chromosome material. This profile shows lower number of chromosomal changes that the number usually detected in RER-cancers. In this case only duplication of chromosome 2 and 19 del were detected.

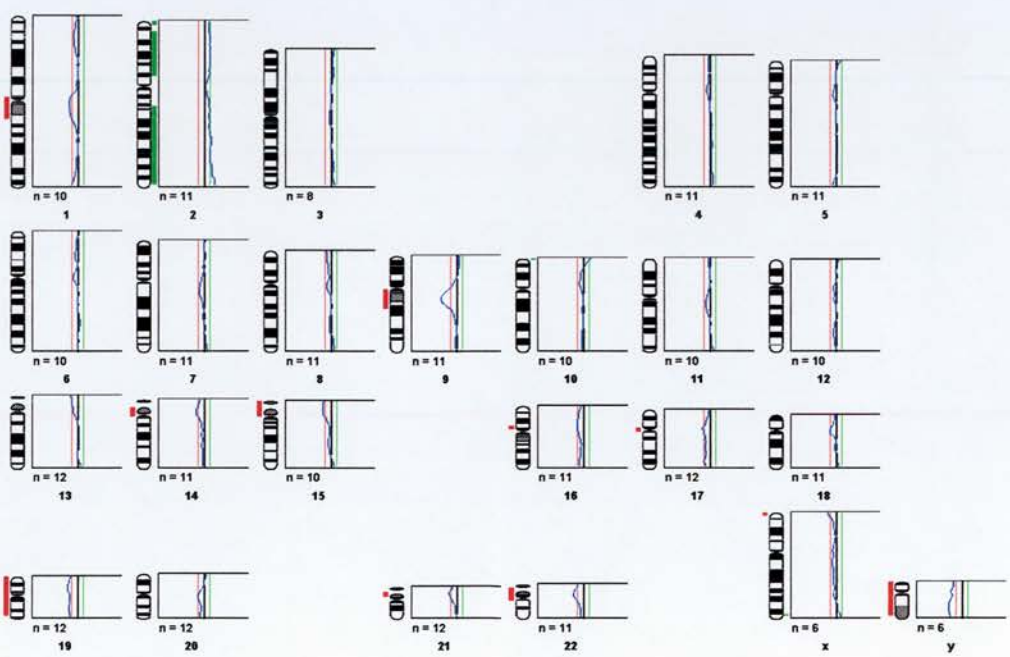


Figure 12. *Chromosome copy number changes as detected by CGH in RER+ colorectal cancers sampled at multiple sites.*

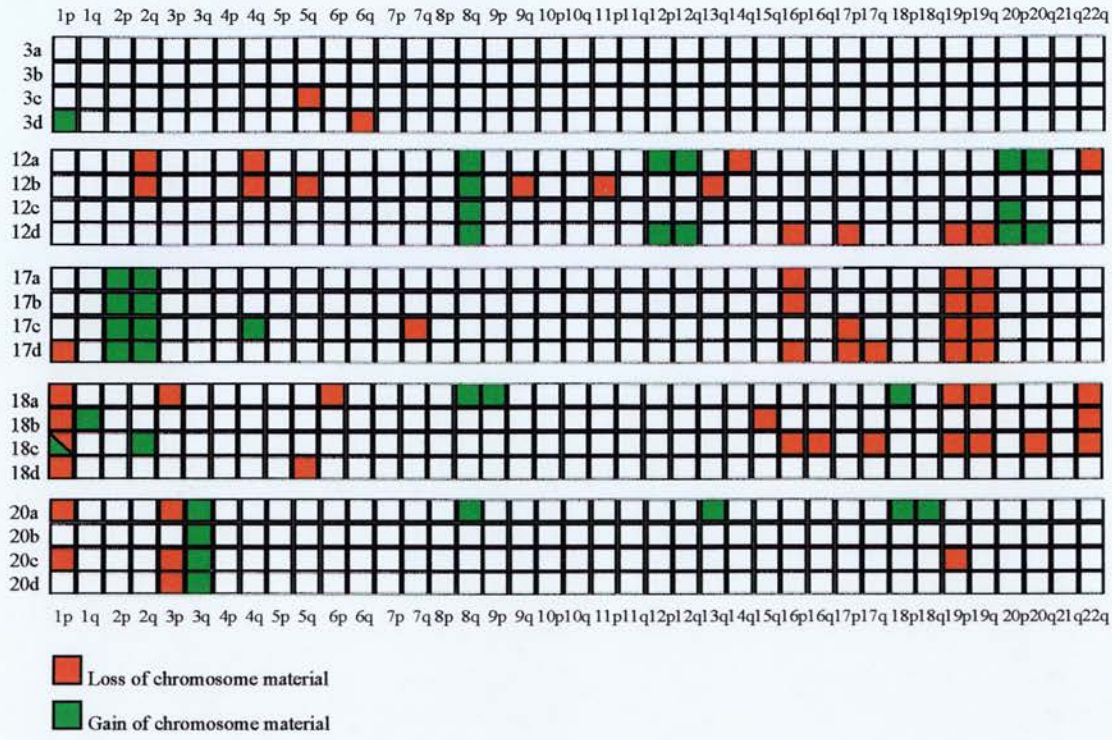
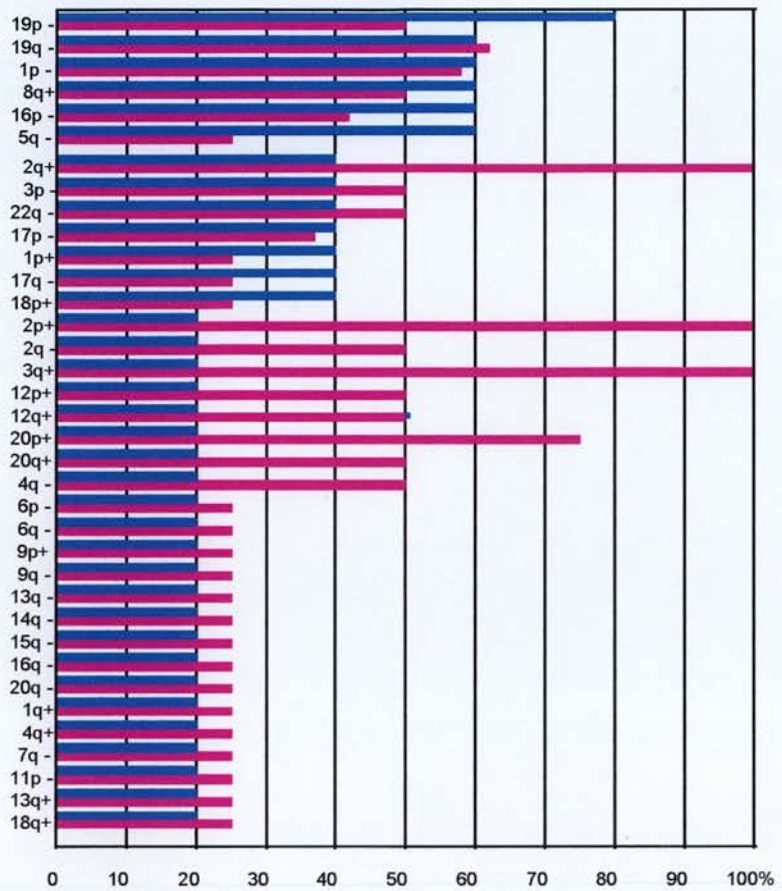


Figure 13. *Frequency of occurrence of chromosomal abnormalities and their prevalence at different tumour sites in RER+ colorectal cancers.*



Frequency of a particular chromosomal change (gain + or loss - of chromosome material) in RER+ colorectal cancers. This number was calculated as a percentage of all RER+ colorectal tumourssexamined in which particular abnormality was observed at any of the multiple sites. examined.

Prevalence of a particular chromosomal change within RER- primary tumours. This number was calculated as the ratio of the number of sites at which a particular chromosomal abnormality was detected and the total number of sites examined averaged over all the tumours where the abnormality occured.

Table 9. *Chromosomal abnormalities most frequently detected in RER+ colorectal cancers.*

Chromosomal changes present in >50% RER+ colorectal cancer cases examined	19p-, 19q-, 1p-, 8q+, 16p-, 5q-
Chromosomal changes present at >50% of different sites examined within a tumour in RER+ colorectal cancer	2p+, 2q+, 19q-, 1p-, 8q+, 19p-
Chromosomal changes present in >50% of tumours at >50% of sites within a tumour in RER+ colorectal cancer	**19p-, 19q-, 1p-**

Chromosomal changes present in >50% of tumours at >50% of sites within a tumour are indicated in colour: loss of chromosome arm - red, gain of chromosome arm - green.

4.3.3. Chromosomal changes are more frequent in RER- than in RER+ colorectal cancers.

The number of chromosomal gains and losses detected by CGH was scored in all RER- and RER+ tumours. The results are presented in Table 10. All statistical analyses were carried out using mean values calculated for each tumour based on the results from multiple sites examined. The analysis revealed that RER- and RER+ colorectal cancers differ not only in the pattern of chromosomal abnormalities most frequently detected but also that the mean frequency of occurrence of chromosomal changes is significantly lower in the RER+ group ($p=0.039$, one-tailed Mann-Whitney Test). Both chromosomal gains and losses are less frequent in RER+ cancers compared to RER- tumours (median 3 and 5 for losses and 2 and 3 for gains respectively) but only the number of chromosomal losses is significantly higher in RER- cancers ($p=0.035$, one-tailed Mann-Whitney Test). Also the loss of chromosomal material is a more frequent event in both groups than chromosome gain although this difference is not statistically significant in either group of tumours (Mann-Whitney Test, $p=0.07$ for RER- and $p=0.46$ for RER+).

Table 10. *Number of chromosomal changes in RER- and RER+ colorectal cancers.*

RER- tumours

ID	No of chrom. losses	M	No of chrom. gains	M	No of chrom changes	M
1a	4		4		8	
1b	6	5	8	7	14	12
1c	7		13		20	
1d	4		2		6	
2a	10		2		12	
2b	17	10	11	6	28	16
2c	7		1		8	
2d	6		10		16	
4a	8		5		13	
4b	7	6	4	4	11	10
4c	5		5		10	
4d	5		4		9	
5a	9		5		14	
5b	0	3	0	2	0	5
5c	1		1		2	
5d	4		2		6	
6a	5		5		10	
6b	8	6	5	4	13	10
6c	6		5		11	
6d	6		3		9	
7a	0		0		0	
7b	6	2	1	0	7	2
7c	0		0		0	
8a	1		0		1	
8b	8	4	2	3	10	7
8c	3		5		8	
8d	4		5		9	
9a	0		1		1	
9b	13	6	4	3	17	9
9c	3		3		6	
9d	7		3		10	
9'a	6	3	2	1	8	4
9'b	1		0		1	
10a	2		0		2	
10b	7	4	1	0	8	4
10c	4		0		4	
11a	12		5		17	
11b	7	11	4	6	11	17
11c	14		11		25	
11d	11		6		17	
13a	4	2	1	1	5	3
13b	1		1		2	
14a	8	6	8	8	16	14
14b	4		8		12	
15a	3	5	0	1	3	6
15b	7		3		10	
16a	2	1	4	5	6	6
16b	0		6		6	
19a	4		1		5	
19b	11	9	4	3	15	12
19c	12		5		17	
22a	4		3		7	
22b	1	5	2	3	3	8
22c	10		4		14	

RER+ tumours

ID	No of chrom. losses	M	No of chrom. gains	M	No of chrom. changes	M
3a	0		0		0	
3b	0	0	0	0	0	0
3c	1		0		1	
3d	1		1		2	
12a	4		5		9	
12b	6	3	1	3	7	6
12c	0		2		2	
12d	4		5		9	
17a	3		2		5	
17b	3	4	2	2	5	6
17c	4		3		7	
17d	6		2		8	
18a	6		3		9	
18b	3	5	1	1	4	6
18c	8		2		10	
18d	2		0		2	
20a	2		5		7	
20b	0	1	1	2	1	3
20c	3		1		4	
20d	1		1		2	

ID, sample ID
M, mean value

4.3.4. Comparison of chromosomal abnormalities present in primary RER- and RER+ colorectal cancers and in corresponding xenografts.

This comparison aimed to establish whether colorectal cancer xenografts are representative of the primary tumour with regard to the assessment of chromosomal abnormalities. Considering the existence of genetic intratumoral heterogeneity within primary tumours, confirmed previously by the study of samples collected from different tumour sites, this study aimed to determine whether the pattern of chromosomal changes detected in the xenografts is influenced by the site of origin of tumour cells used for establishing xenografts.

Some discrepancies between the CGH results for primary tumours and corresponding xenografts were expected, since such differences were detected within primary tumours. It was important, however, to establish whether despite these discrepancies, a similar pattern of chromosomal changes to that detected in RER- and RER+ primary colorectal cancers was present in xenografts derived from these tumours and whether they were therefore representative of their tumours of origin.

An attempt was made to establish xenografts from all collected tumour samples. Out of 76 samples implanted in SCID mice 27 were successfully established. This included 15 xenografts derived form 6 RER- cancers and 12 xenografts derived from 4 RER+ tumours. Two of these xenografts had to be subsequently excluded from the analysis for the reasons discussed in chapter 6. In two further cases CGH data could not be obtained, despite numerous attempts.

Figures 14 and 15 present the results of CGH analysis of RER- and RER+ primary tumours sampled at multiple sites and the corresponding xenografts.

In both groups, more chromosomal changes were found in colorectal cancer xenografts compared to the primary tumours. This was as expected, since DNA extracted from primary tumours was contaminated to some degree with normal DNA reducing CGH sensitivity. Tables 11a and 11b present mean numbers of chromosomal gains and losses detected by CGH in relevant samples from the primary tumours and in the corresponding xenografts.

Table 11a. *Mean number of chromosomal gains and losses detected by CGH in primary RER- tumours and in corresponding xenografts. The last column indicates the proportion of chromosomal changes detected in the primary tumours compared to the xenografts.*

Primary tumours				Xenografts				%
Tumour ID	Losses	Gains	Changes	Xenograft ID	Losses	Gains	Changes	
1	5	7	12	***1x***	13	14	27	44%
4	7	4	11	***4x***	9	10	19	58%
5	3	2	5	***5x***	12	4	16	31%
19	11	4	15	***19x***	16	8	24	62%
22	5	3	8	***22x***	18	7	25	32%

Values in the last column are calculated as an indication of what proportion of changes detected in the corresponding xenografts was detected in the primary tumour. These calculations are based purely on the number of changes.

Table 11b. *Mean number of chromosomal gains and losses detected by CGH in primary RER+ tumours and in corresponding xenografts. The last column indicates the proportion of chromosomal changes detected in the primary tumours compared to the xenografts.*

Primary tumours				Xenografts				%
Tumour ID	Losses	Gains	Changes	Xenograft ID	Losses	Gains	Changes	
12	3	3	6	***12x***	6	3	9	67%
18	7	2	9	***18x***	8	4	12	62%
20	2	2	4	***20x***	6	3	9	44%

Values in the last column are calculated as an indication of what proportion of changes detected in the corresponding xenografts was detected in the primary tumour. These calculations are based purely on the number of changes.

Chromosomal abnormalities present in primary tumours were usually detected in corresponding xenografts (see Table 12). This rule applied especially to the RER- colorectal cancers, where in general 75% of the chromosomal changes present in the primary tumours were also detected in the corresponding xenografts, compared with 56% in RER+ matched pairs.

The presence of chromosomal changes characteristic for the RER- colorectal cancer group was highly consistent between primary tumours and corresponding xenografts (see Table 13). Where changes typically found in RER- cancers (20q+, 18q-13q+, 8p-, 1p- and 8q+) were detected in the sample from the primary tumour, 92% were also present in the corresponding xenograft. This was not found to be the case in the

RER+ tumours where the consistency of finding chromosomal abnormalities typical of RER+ tumours (19p-, 19q-, 1p-) reached only 57%.

Table 12. *The proportion of chromosomal changes found in primary tumours that were also detected in corresponding xenografts.*

RER-		**RER+**	
Primary tumour/xenografts	%	Primary tumour/ xenograft	%
1a/1xa	100	***12a/12xa***	55
1b/1xb	71	***12b/12xb***	14
1c/1xc	45	***12c/12xc***	100
1d/1xd	60	***12d/12xd***	100
4b/4xb	81	***18a/18xa***	20
		18c/18xc	0
5a/5xa	50	***20a/20xa***	43
5b/5xb	-	***20b/20xb***	75
5c/5xc	100	***20c/20xc***	100
19b/19xb	77		
19c/19xc	76		
22a/22xa	86		
22b/22xb	67		
22c/22xc	86		

-, indicates that there were no chromosomal changes present in the primary tumour, rendering the score impossible.

Table 13. *The consistency of chromosomal changes characteristic of RER- (20q+, 18q-, 13q+, 8p-, 1p- and 8q+) and RER+ (19p-, 19q-, 1p-) tumours appearing in primary colorectal cancers and corresponding xenografts.*

RER-		**RER+**	
Primary tumour/xenografts	%	Primary tumour/ xenograft	%
1a/1xa	100	***12a/12xa***	-
1b/1xb	100	***12b/12xb***	-
1c/1xc	75	***12c/12xc***	-
1d/1xd	100	***12d/12xd***	100
4b/4xb	80	***18a/18xa***	0
		18c/18xc	33
5a/5xa	100	***20a/20xa***	100
5b/5xb	-	***20b/20xb***	50
5c/5xc	100	***20c/20xc***	-
19b/19xb	100		
19c/19xc	100		
22a/22xa	100		
22b/22xb	50		
22c/22xc	100		

-, indicates that there were no characteristic chromosomal changes present in the primary tumour, rendering the score impossible.

Figure 14. *Chromosomal abnormalities detected by CGH in RER- colorectal cancers sampled at multiple sites and in the corresponding xenografts.*

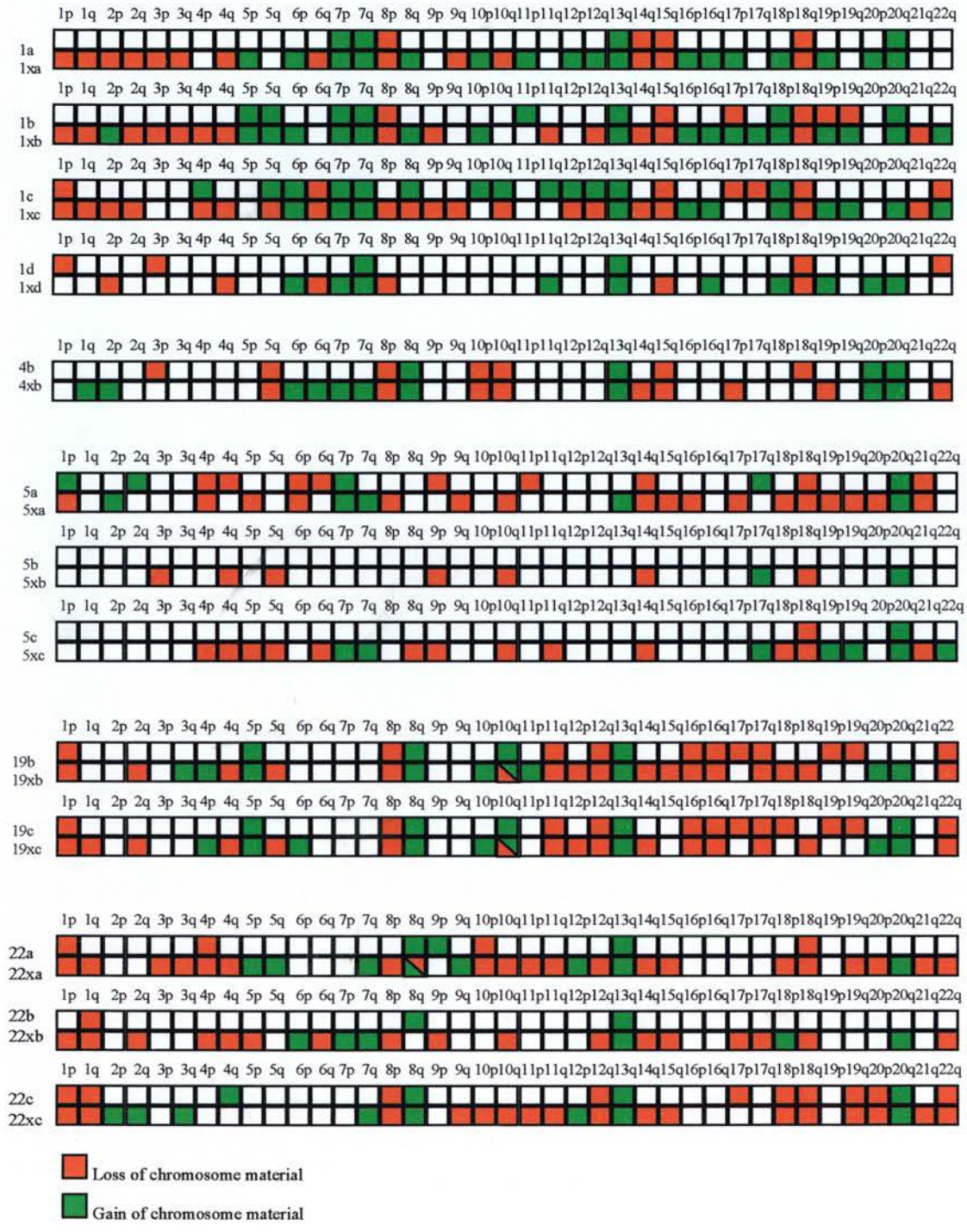
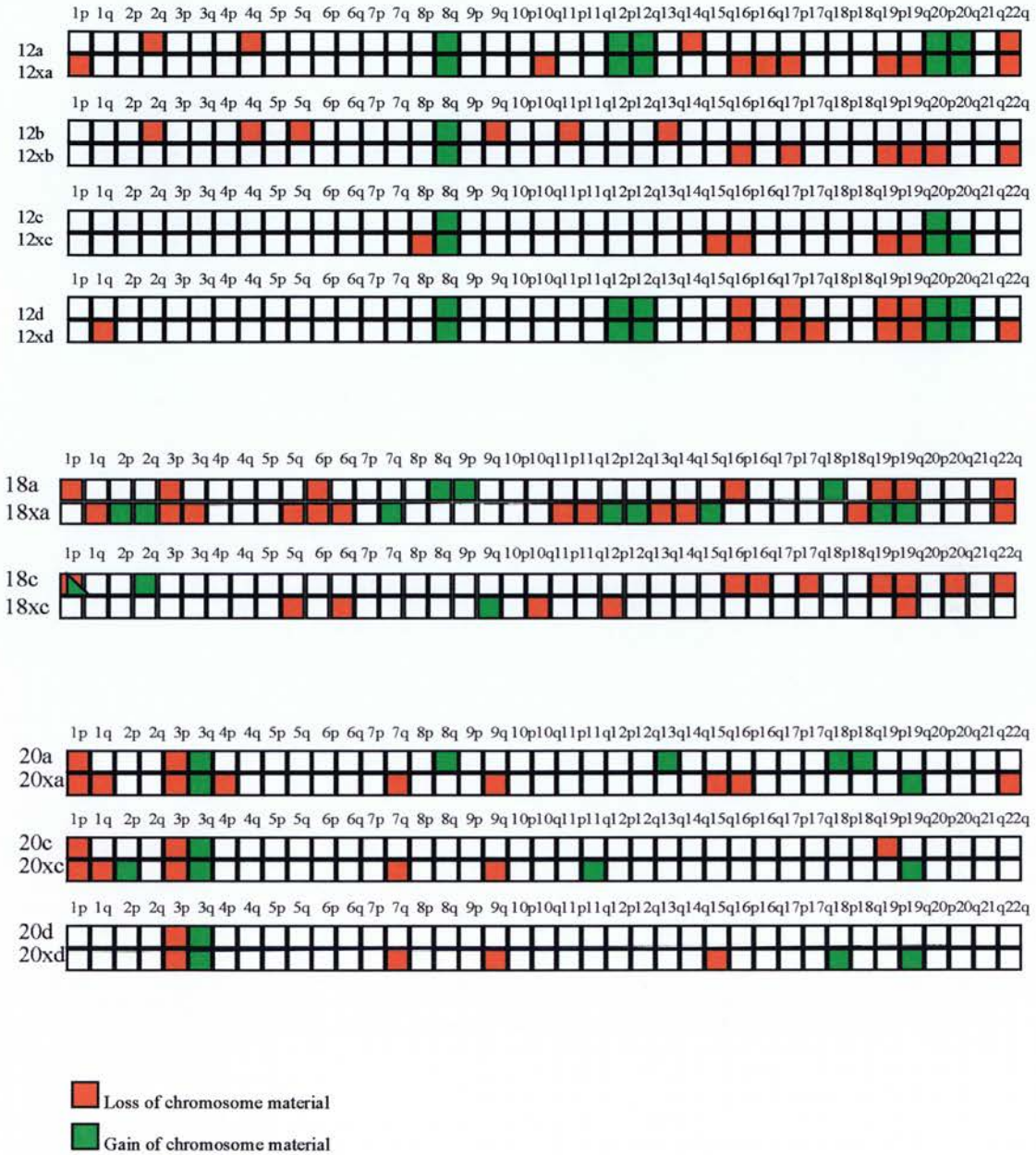


Figure 15. *Chromosomal abnormalities detected by CGH in RER+ colorectal cancers sampled at multiple sites and in the corresponding xenografts.*



4.3.5. Assessment of the level of chromosomal instability in RER- and RER+ colorectal cancers based on the CGH data.

Although the presence of multiple abnormalities in chromosome number or structure is often taken to represent chromosomal instability, the possibility also exists that tumour karyotypes represent a new stable equilibrium, adopted after an earlier phase (or perhaps even single episode) of violent change. To distinguish between these possibilities two different indices were used. The first was a single count of the number of gains or losses per genome. In CGH, this number is always referred back to the standard diploid karyotype, regardless of the total ploidy of the tumour genome, since equivalent quantities of tumour and normal DNA are cohybridised with the target standard metaphase chromosomes. Second index sought to express numerically the extent to which samples from different parts of the tumour displayed similar or dissimilar features. Once again this index is referenced against the normal karyotype, so each chromosome arm contributes equally to the analysis. Thus chromosome arms in which the CGH pattern is identical for each sampled portion of the tumour score zero, whilst arms for which the sampled portions differ from each other score unity (for example see Figure 16). Although it is recognised that this index may be sensitive to the number of sites sampled per tumour, there is no *a priori* reason to suppose that the heterogeneity index and the chromosome number index should measure the same properties. This was the basis of the decision to adopt as CIN index the sum of the two.

In practice, however, heterogeneity index and number of chromosome changes show a modest positive correlation (Figure 17). The correlation coefficient was 0.622 and the significance 0.002 (two-tailed Spearman rank correlation coefficient test). Thus it is probable that the clonal evolution of these tumours is continuous - tumours with highest divergence also showing the highest total number of changes. The only exceptions appear to be tumour No14 and No19, both of which have low heterogeneity indices despite high numbers of altered chromosomes. Since, however, both were analysed on the basis of two and three separate portions respectively (rather than 4, as in the majority of tumours analysed), the heterogeneity index may have been underestimated.

Figure 16. CGH results of chromosomal gains and losses detected in a primary tumour sampled at 4 different sites and in 4 corresponding xenografts.
CGH results are shown for the tumour from patient no.1 as an example of how the 'heterogeneity score' was calculated for each patient. Chromosome arms are represented vertically in columns, whilst each different site of the same tumour designated a, b, c and d are in rows. 1xa, 1xb, 1xc, and 1xd are the corresponding xenografted tumours established from sites a, b, c and d.
The heterogeneity score was calculated for the primary tumour and separately for the xenograft by adding the number of columns representing chromosome arms in which changes were inconsistent between different sites within the tumour. For example, in the primary tumour, inconsistent chromosome changes are seen in 1p, 3p, 4p, 5p, 5q, 6p, 6q, 7p, 8p, 8q, 10p, 10q, 11p, 11q, 12p, 12q, 14q, 15q, 17p, 17q, 18p, 19p, 19q, 20q and 22q giving a total heterogeneity score of 25. Consistent patterns, such as at 1q or 7q, do not score.

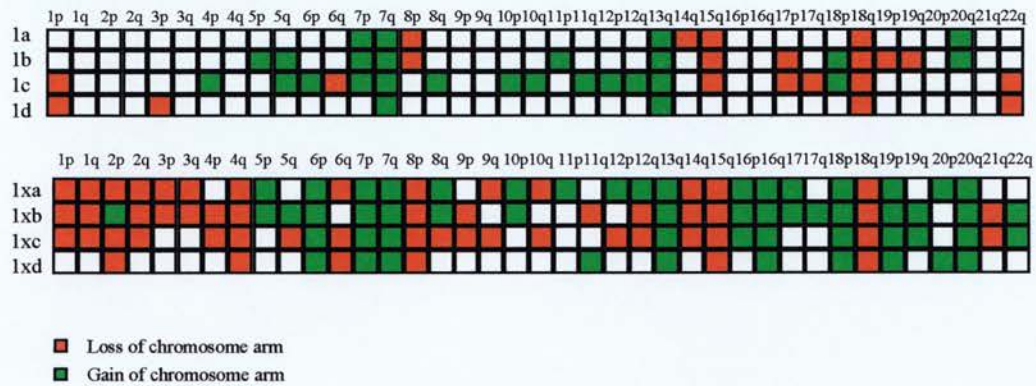


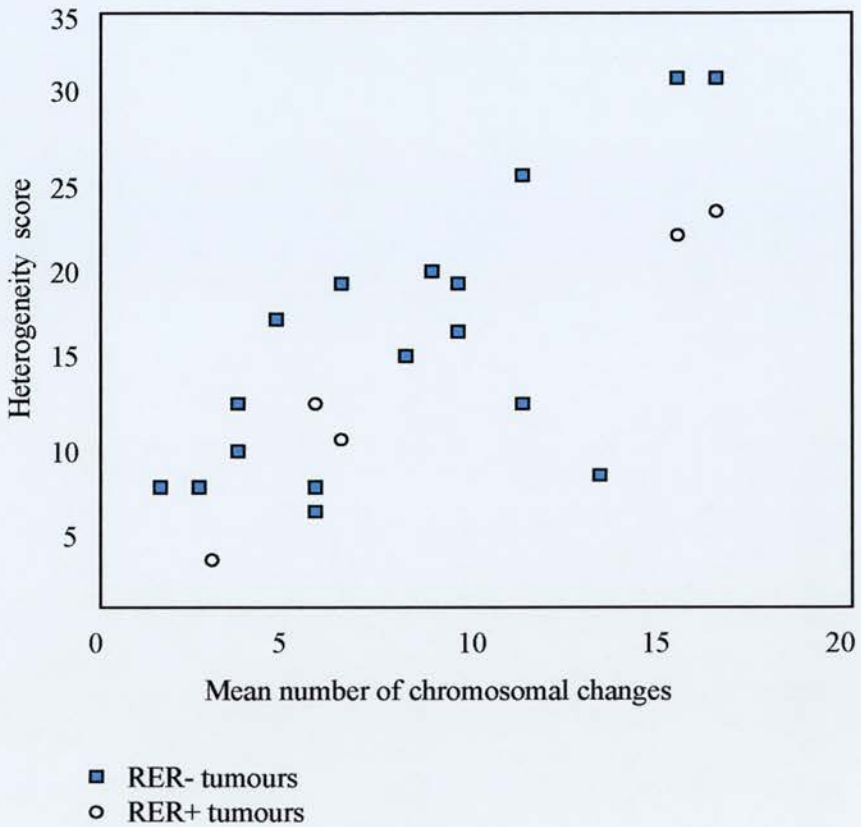
Table 14 presents the scores of the mean number of chromosomal changes, heterogeneity scores and the combined CIN index scores in relation to the tumours' RER status.

As previously mentioned, the mean number of chromosomal gains and losses was significantly higher in RER- colorectal cancers compared to RER+ tumours (one-tailed Mann-Whitney Test, p=0.039). Heterogeneity of chromosomal changes also appeared higher in RER- cancers, though this did not reach significance (one-tailed Mann-Whitney Test, p=0.068). The combined score (CIN index), however, was again significantly higher in RER- than in RER+ tumours (one-tailed Mann-Whitney Test, p=0.039).

Table 14. *Chromosomal instability index in RER- and RER+ colorectal cancers.*

RER-				RER+			
Tumour ID:	Mean number of chromosomal changes	Heterogeneity score	CIN index	Tumour ID:	Mean number of chromosomal changes	Heterogeneity score	CIN index
1	12	25	37	***3***	0	3	3
2	16	31	47	***12***	6	16	22
4	10	16	26	***17***	6	6	12
5	5	17	22	***18***	6	17	23
6	10	19	29	***20***	3	7	10
7	2	7	9				
8	7	19	26				
9	9	20	29				
9'	4	9	13				
10	4	12	16				
11	17	31	48				
13	3	7	10				
14	14	8	22				
15	6	7	13				
16	6	6	12				
19	12	12	24				
22	8	15	23				
	p=0.039	**p=0.068**	**p=0.039**	Probability that mean values for RER- are greater than for RER+			

Figure 17. *Mean number of chromosomal changes plotted against heterogeneity score shows correlation of these two values.*



The correlation coefficient 0.622, the significance 0.002 (two-tailed Spearman rank correlation coefficient test)

4.3.6. Comparative studies identify a subgroup of RER- colorectal cancer exhibiting a low level of chromosomal instability.

Further data analysis showed that although RER- tumours compared to RER+ cancers show an overall significantly higher level of chromosomal instability there is a group of RER- colorectal cancers with quite a low level of CIN comparable to that observed in RER+ cancers (see Figure 18). This group of RER- tumours fell below a CIN index of 18 , a subjectively defined borderline between the two groups, which is a middle value between mean CIN index values for all RER- and all RER+ tumours.

This surprising finding suggests that low CIN subgroup of RER- cancers may represent separate subclass distinct from high-CIN tumours, since it shows neither microsatellite instability (as RER+ cancers) nor major repeated changes in chromosome number (as high-CIN RER- cancers). This non-MIN non-CIN group of

colorectal cancer quite possibly represents a distinct entity and might harbour none or yet another mechanism allowing destabilisation of the genome.

The question therefore arises if there are clues to what this mechanism might be. One immediate possibility is a different pattern of alteration of critical "caretakers" (genes responsible for stability of the genome), potentially leading to different patterns of genomic instability.

To further investigate this subset of tumours, any differences in the patterns of specific clonal chromosomal abnormalities present in low and high CIN RER- cancers were analysed.

Chromosomal gains and losses most frequently present in high CIN RER- cancers (20p+, 18q-, 13q+, 8p-, 1p-, 8q+) (see Table 8) were consistently less common in the group of colorectal cancers with low CIN (Table 15). Particularly striking was the lack of incidence of 13q duplication in RER- tumours with low CIN (detected in 1 out of 6 tumours) compared with RER- cancers with a high level of chromosomal instability (10 out of 11 tumours). Although this finding was not entirely surprising, since this group exhibited an overall lower level of chromosomal instability, it was more interesting in light of the fact that not all chromosomal changes were less common in low CIN RER- cancers. Surprisingly deletions of all or part of chromosome 19, one of the two frequent chromosomal changes in RER+ cancers in our study (see section 4.3.2), appeared with similar frequency in low and high CIN RER- tumours (occurring in 3/6 low CIN and 5/11 high CIN RER- tumours).

Table 15. *Distribution of the most frequent chromosomal changes detected in RER- colorectal cancers with regard to CIN level.*

	All RER- tumours	**RER- tumours with CIN>18**	**RER- tumours with CIN<18**
Total number of tumours in each group:	**17**	**11**	**6**
Most frequent chromosomal abnormalities in RER- tumours:			
20q+	88% (15)	100% (11)	67% (4)
18q-	76% (13)	91% (10)	50% (3)
13q+	65% (11)	91% (10)	17% (1)
8p-	59% (10)	73% (8)	33% (2)
8q+	53% (9)	64% (7)	33% (2)
1p-	53% (9)	64% (7)	33% (2)

4.3.7. Clinicopathological features of RER- tumours with low levels of chromosomal instability.

Overall, this entire series consisted of 8 right-sided and 14 left-sided tumours (see Appendix 1a).

An attempt was made to establish whether the group of RER- colorectal cancers exhibiting low levels of chromosomal instability might constitute a distinctive entity based on clinicopathological features. All of the 6 RER- sporadic colorectal cancers with low levels of CIN were left-sided compared with 8 of 11 high-CIN RER- tumours. Four of the tumours were moderately differentiated adenocarcinomas but well and poorly differentiated tumours were also observed. There was no difference between the proportion of low CIN and high CIN cancers with regard to Dukes' stage or patient age. Thus there appeared to be no substantial clinicopathological differences between high and low CIN RER- tumours. Significantly, however, the low CIN RER- tumours differed radically in location from the RER+ tumours, which also included low CIN cancers, but which were all right sided.

4.3.8. Progress of genomic instability in CIN colorectal cancers reflected by genomic differences between primary tumours and corresponding xenografts.

Passage of tumour cells derived from primary RER- and RER+ colorectal cancers as xenografts in SCID mice allowed the assessment of the temporal evolution of chromosomal abnormalities in the examined tumours and thus assessment of the level of underlying chromosomal instability.

To assess the progress of chromosomal changes in these tumours, a scoring system similar to that for primary tumours was applied. For this purpose only those xenografts successfully established from at least two separate sites from the primary tumour were used, since this was a prerequisite for calculating the heterogeneity score. The assumption was made that if chromosomal instability is an underlying mechanism driving tumorigenesis in a proportion of tumours, the CIN index should be even higher in the corresponding xenografts. On the other hand, if the chromosomal changes represented accidental events, the CIN index should not differ dramatically between primary tumours and corresponding xenografts.

The fact that the same cut-off point of 1.125 an 0.875 in CGH analysis was used for scoring chromosomal gain and loss in primary tumours and in xenografts could result in possible underscoring of chromosomal changes in the primary tumours, which were contaminated to some degree with normal stromal DNA. However, since the same error applied to both RER- and RER+ groups of colorectal cancer, direct comparison of the results could be made.

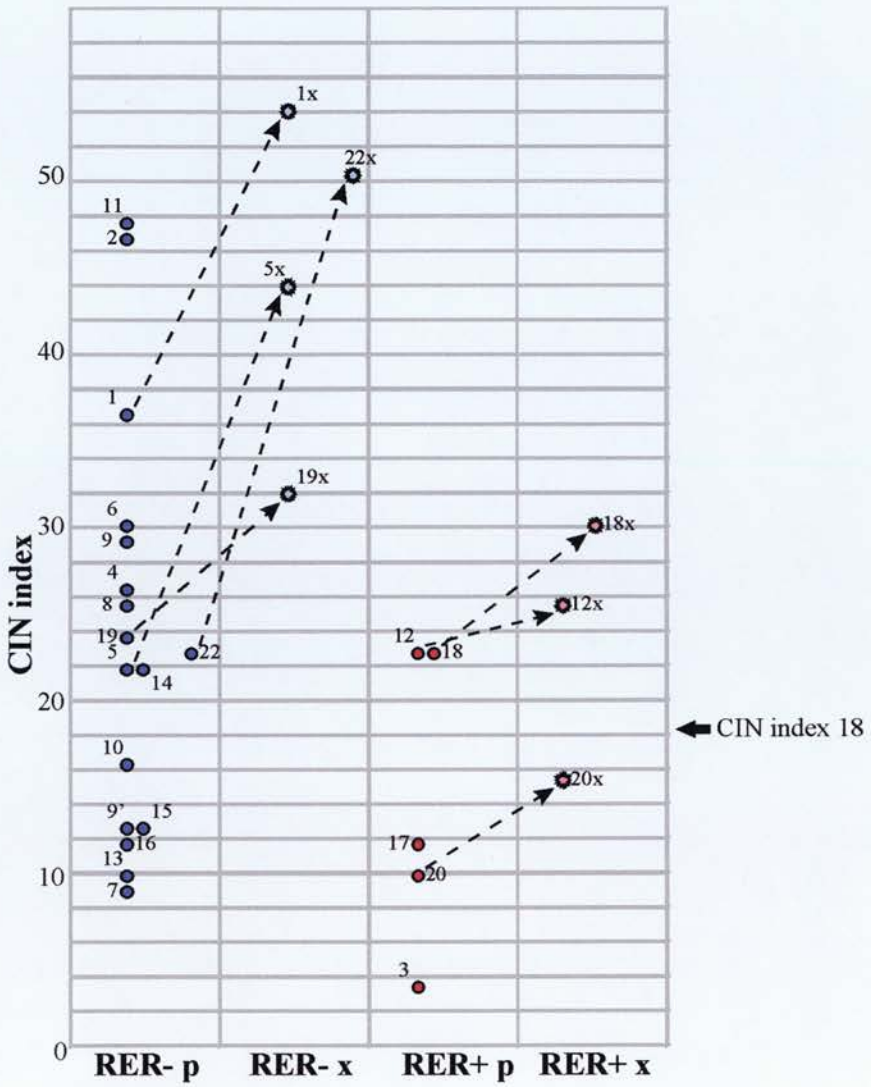
Table 16 presents the results.

Table 16. *Chromosomal instability index in RER- and RER+ colorectal cancer xenografts.*

RER- xenografts					**RER+ xenografts**				
Xenograft ID	Mean number of chromosomal changes	Heterogeneity score	CIN index	Average xenograft age (weeks)	Xenograft ID	Mean number of chromosomal changes	Heterogeneity score	CIN index	Average xenograft age (weeks)
1x	27	27	54	15	***12x***	10	16	26	4.5
5x	17	27	44	12	***18x***	13	17	30	12.5
19x	25	7	32	12	***20x***	9	7	16	11
22x	26	24	50	9.5					

The data showed substantial differences in the degree to which CIN index increased between primary tumours and xenografts of RER- and RER+ cancers (74% and 34% respectively), reflecting in general an increased rate of acquisition of chromosomal abnormalities in RER- tumours compared to RER+ cancers (see Figure 18).

Figure 18. *CIN index in RER- and RER+ primary tumours and in the corresponding xenografts. The figure illustrates substantially greater increase of CIN index in RER- group of tumours.*



RER-p, RER- primary tumours
RER-x, RER- xenografts
RER+ p, RER+ primary tumours
RER+ x, RER+ xenografts

4.3.9. Flow cytometry analysis of the DNA content in RER- and RER+ colorectal cancers sampled at multiple sites and in the corresponding xenografts.

Following the CGH analysis, the DNA content of all samples included in this study was re-examined using flow cytometry to test whether the results gained by both methods were compatible and to provide additional information on the tumour cells' total DNA content. Samples collected at multiple sites from primary tumours were examined to determine whether DNA content of tumour cells was consistent within the tumour or varied between different tumour sites. Samples from corresponding xenografts were analysed in order to determine whether DNA content of tumour cells was temporally stable.

The results are presented in Table 17 and 18.

Table 17. *Flow cytometry analysis of the DNA content of RER+ tumours sampled at multiple sites and of the corresponding xenografts.*

Primary tumours			**Xenografts**		
Sample ID	DNA content	DNA index	Xenograft ID	DNA content	DNA index
3a	diploid		-		
3b	diploid		-		
3c	diploid		-		
3d	diploid		-		
12a	diploid		*12xa*	diploid	
12b	diploid		*12xb*	diploid	
12c	diploid		*12xc*	diploid	
12d	diploid		*12xd*	diploid	
17a	diploid		-		
17b	diploid		-		
17c	diploid		-		
17d	diploid		-		
18a	near tetraploid	1.93	*18xa*	near tetraploid	1.83
18b	near tetraploid	1.95	*18xb*	near tetraploid	1.93
18c	diploid		*18xc*	diploid	
18d	near tetraploid	1.87	*18xd*	near tetraploid	1.98
20a	diploid		*20xa*	diploid	
20b	diploid		-		
20c	diploid		*20xc*	diploid	
20d	diploid		*20xd*	diploid	

The DNA index was calculated as the ratio of peak positions of the aneuploid and diploid peaks. DNA index values are given for peaks with cell content exceeding 10% of the total cell count.
Samples were classified as near diploid or near tetraploid if the tumour cells' peak fluorescence value was within 10% of the diploid or tetraploid peak fluorescence value, but for statistical purposes were treated as aneuploid.
- , not available

Table 18. *Flow cytometry analysis of the DNA content of RER- tumours sampled at multiple sites and of the corresponding xenografts.*

Primary tumours			Xenografts		
Sample ID	DNA content	DNA index	Sample ID	DNA content	DNA index
1a	near tetraploid	1.82	*1xa*	near tetraploid	1.85
1b	near tetraploid	1.84	*1xb*	near tetraploid	1.89
1c	near tetraploid	1.84	*1xc*	near tetraploid	1.80/2.05
1d	near tetraploid	1.82	*1xd*	near tetraploid	1.84
2a	near diploid	1.1	-		
2b	near tetraploid	2.2	-		
2c	n.dipl./n.tetrapl.	1.2/2.2	-		
2d	near diploid	1.1	-		
4a	aneuploid	1.54	-		
4b	aneuploid	1.6	*4xb*	aneuploid	1.94
4c	aneuploid	1.59	-		
4d	aneuploid	1.58	-		
5a	aneuploid	1.65	*5xa*	aneuploid	1.75
5b	aneuploid	1.65	*5xb*	aneuploid	1.64
5c	aneuploid	1.58	*5xc*	aneuploid	1.62
5d	aneuploid	1.63	-		
6a	aneuploid	1.66	-		
6b	aneuploid	1.68	-		
6c	aneuploid	1.72	-		
6d	aneuploid	1.68	-		
7a	near tetraploid	1.81	-		
7b	aneuploid	1.76	-		
7c	near tetraploid	1.85	-		
8a	aneuploid	1.68	-		
8b	aneuploid	1.73	*8xb*	aneuploid	1.75
8c	aneuploid	1.76	-		
8d	aneuploid	1.65	-		
9a	near tetraploid	1.87	-		
9b	near tetraploid	1.92	-		
9c	near tetraploid	1.96	-		
9d	near tetraploid	2.04	-		
9a'	diploid		-		
9b'	diploid		-		
10a	diploid		-		
10b	diploid		-		
10c	diploid		-		
11a	aneuploid	1.43	-		
11b	near tetraploid	2.05	-		
11c	aneuploid	1.45	-		
11d	aneuplod	1.36	-		
13a	diploid		-		
13b	diploid		-		
14a	near diploid	1.1	-		
14b	diploid		-		
15a	aneuploid	1.4	-		
15b	diploid		-		
16a	diploid		-		
16b	diploid		-		
19a	aneuploid	1.54	-		
19b	aneuploid	1.54	*19xb*	aneuploid	1.62/3.2
19c	aneuploid	1.52	*19xc*	aneuploid	1.62
22a	aneuploid	1.32/2.43	*22xa*	aneuploid	1.34/2.6
22b	aneuploid	1.32/2.3	*22xb*	near tetraploid	1.8
22c	aneuploid	1.4/2.3	*22xc*	aneuploid	1.42

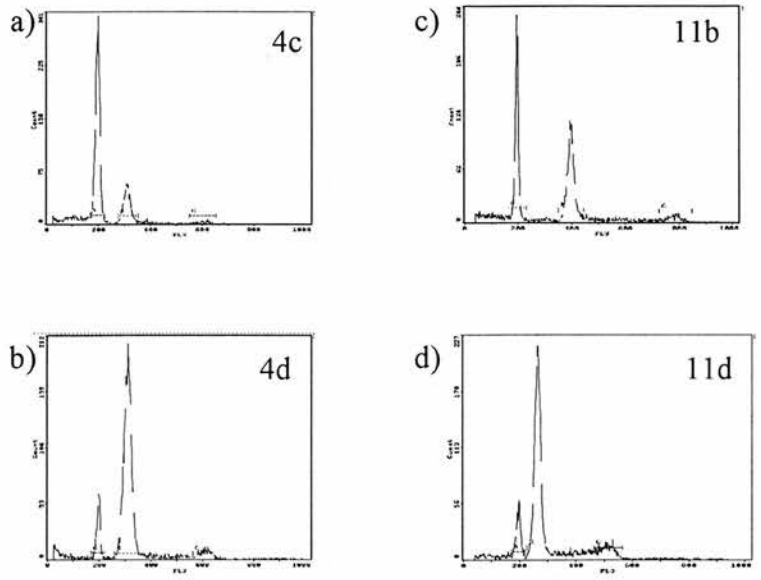
The DNA index was calculated as the ratio of peak positions of the aneuploid and diploid peaks. DNA index values are given for peaks with cell content exceeding 10% of the total cell count.
Samples were classified as near diploid or near tetraploid if the tumour cells' peak fluorescence value was within 10% of the diploid or tetraploid peak fluorescence value, but for statistical purposes were treated as aneuploid.
- , not available

Similar to the results of the CGH analysis which revealed that fewer chromosomal changes are present in RER+ tumours compared to RER- cancers, flow cytometry analysis of the total DNA content showed most of the RER+ colorectal tumours (4 out of 5) to have diploid DNA content, whilst in RER- cancers the majority of tumours (13 out of 17) were aneuploid. This difference was statistically significant (two-tailed Fisher exact test, $p=0.039$). Moreover, all but one of the 6 RER- cancers with a diploid clone were classed as non-MIN non-CIN tumours whilst only one of the 6 non-MIN non-CIN RER- cancers was aneuploid.

Interestingly many of the RER- tumours exhibited intratumoral heterogeneity of the DNA content with DNA indexes varying in samples taken from different tumour sites (see Figure 19). This phenomenon was present in only one RER+ cancer and the four remaining tumours retained their diploid DNA content within the tumour and in the corresponding xenografts (see Figure 20).

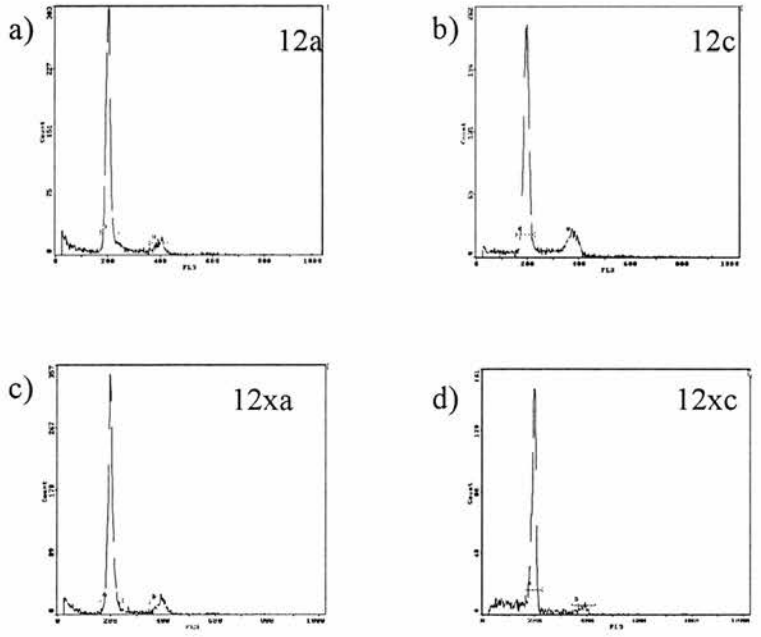
Change in the total DNA content with time could be observed in RER- colorectal cancers, where many of the corresponding xenografts showed a somewhat different DNA content to the tumour sample from which they were established (see figure 21). However colorectal cancer xenografts established from tumours with diploid DNA content always remained diploid and those established from aneuploid tumours remained aneuploid.

Figure 19. *Flow cytometry profiles of the DNA content of two RER- primary colorectal cancers sampled at multiple sites.*



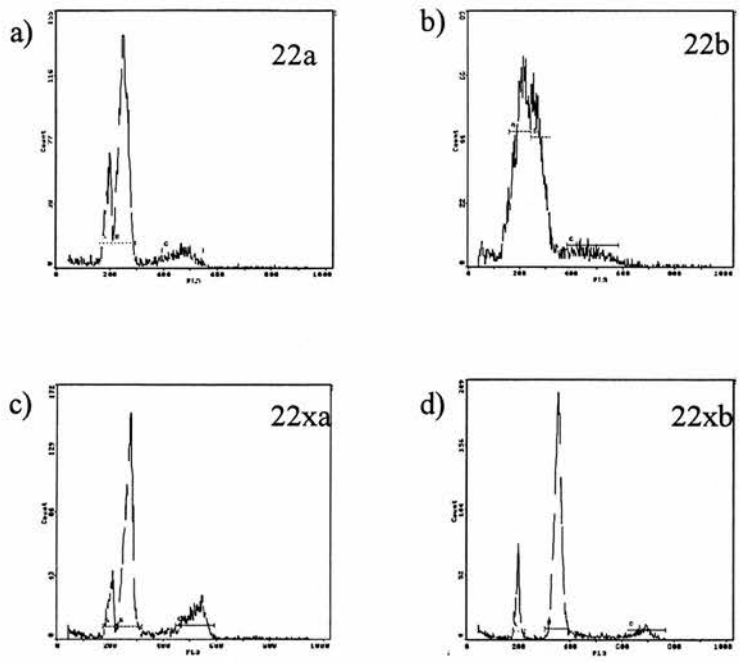
a) and b) show flow cytometry profiles for two sites within RER- tumour No4 (4c and 4d) with similar aneuploid peak positions
c) and d) show flow cytometry profiles for two sites within RER- tumour No11 (11b and 11d) with substantially different aneuploid peak positions

Figure 20. *Flow cytometry profiles of the DNA content of a RER+ colorectal cancer sampled at multiple sites and of the corresponding xenografts.*



a) and b) show flow cytometry profiles for two sites within diploid RER+ tumour No 12 (12a and 12c)
c) and d) show flow cytometry profiles of xenografts established from samples 12a and 12c which retained their diploid DNA content

Figure 21. *Flow cytometry profiles of the DNA content of a RER- colorectal cancer sampled at multiple sites and of the corresponding xenografts.*



a) and b) show flow cytometry profiles for two sites within aneuploid RER- tumour No 22 (22 a and 22b)
c) and d) show flow cytometry profiles of xenografts established from samples 22a and 22b which remained aneuploid, despite changing their DNA content compared to their samples of origin

4.4. Discussion.

4.4.1. Frequency of chromosomal abnormalities in RER- and RER+ sporadic colorectal cancers.

Differences in the frequency of chromosomal abnormalities in RER- and RER+ colorectal cancers have been previously reported. RER+ tumours have been shown to retain their diploid DNA content (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993). Studies of chromosomal abnormalities in RER- and RER+ colorectal cancers cell lines indicated chromosomal gains and losses to be substantially less common in RER+ tumours (Schlegel *et al.*, 1995; Eshleman *et al.*, 1998a). The results of this study are in concordance with these findings.

Differences in the frequency of occurrence of chromosomal changes in RER- and RER+ groups of colorectal cancer support the hypothesis that two distinct mechanisms drive carcinogenesis in these two colorectal cancer groups.

Although the incidence of chromosomal instability is significantly higher in the RER- colorectal cancers compared to RER+ tumours, the results of our study suggest that CIN is probably not the sole mechanism responsible for cancer development in the RER- group of sporadic colorectal cancer.

Unlike the results of Eshleman *et al.* (1998a), which indicated that RER- and RER+ colorectal cancer cell lines could be clearly distinguished on the basis of chromosomal instability index (which they defined as the sum of all non-disjunction events and all chromosomal breaks) we observed substantial variation in levels of chromosomal abnormalities between the two groups. Methods and scoring systems used in these two studies were not identical but both reflected the level of chromosomal instability in the tumours examined. These results were confirmed by the flow cytometry analysis of the total DNA content. Most of the MIN and non-MIN non-CIN cancers appeared to retain the diploid DNA content.

4.4.2. Identification of a novel non-MIN, non-CIN phenotype in sporadic colorectal cancer.

This study has identified a significant proportion of sporadic colorectal cancers, up to one quarter, which do not display instability of either chromosomes or microsatellites. These tumours do not to show any striking differences in clinical and pathological features to high CIN RER- tumours but may harbour fewer abnormalities of p53 (see Chapter 5). The data also suggest that these cancers might display a different pattern of clonal chromosomal abnormalities to high CIN RER- tumours. More frequent loss of chromosome 19, despite infrequent chromosomal abnormalities together with rare defects of p53 might indicate that a different subset of tumour suppressor genes is targeted in these tumours. Interestingly mutations in *STK11*, a recently identified tumour suppressor gene (Peutz-Jeghers gene) mapped to 19p13.3 (Hemminki *et al.*, 1997) were found only in left sided colorectal cancers (Dong *et al.*, 1998). All non-MIN non-CIN colorectal cancers identified in this study were left sided tumours.

Failure to distinguish this group of tumours prior to this study may be due to the heavy reliance of many investigators on tumours established as cell cultures and under-representation of non-MIN non-CIN cancers among colorectal cancer cell lines (Eshleman *et al.*, 1998a). Although we have not attempted to establish cell lines from the tumours in this study we did, unusually, fail to establish xenografts from all six of the non-MIN non-CIN tumours, despite implanting multiple samples from each. The other colorectal cancers in this series were implanted with a success rate of 36% for RER- and 60% for RER+.

4.4.3. Patterns of chromosomal abnormalities in RER- and RER+ colorectal cancers.

4.4.3.1. Specific pattern of chromosomal abnormalities in RER- sporadic colorectal cancers.

The specific pattern of chromosomal gains and losses (20q+, 18q-, 13q+, 8p-, 1p-, 8q+) identified in the 17 RER- sporadic colorectal cancers included in this study generally conforms with the results of many previous cytogenetic and CGH studies (Reichmann *et al.*, 1981; Muleris *et al.*, 1985, 1988, 1990, 1994; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Xiao *et al.*, 1992; Bardi *et al.*, 1993a, 1993b, 1995; Barletta *et al.*, 1993; Herbergs *et al.*, 1994; Gerdes *et al.*, 1995; Bomme *et al.*, 1996; Herbergs *et al.*, 1996; Ried *et al.*, 1996; Mertens *et al.*, 1997). The only exception is duplication of chromosome 7, previously reported as a frequent and early event in colorectal carcinogenesis (Griffin *et al.*, 1993; Muleris *et al.*, 1994; Bomme *et al.*, 1996; Herbergs *et al.*, 1996). In this study 7p+ and 7q+ was found in 53% and 35% of RER- colorectal cancers respectively but gain of the whole chromosome 7 was present in less than 50% of RER- tumours and was not detected at all in RER+ cancers. The results also do not confirm gain of chromosome 7 to be an early event in colorectal cancer development, since its distribution within tumours was rather heterogeneous.

CGH analysis of chromosome abnormalities at different tumour sites in RER- and RER+ sporadic colorectal cancers allowed the identification of chromosomal changes that are most likely specifically selected for in these groups of tumours. Frequent loss of the long arm of chromosome 18 in RER- cancers could be explained by the fact that tumour suppresser genes such as *DCC* (Itoh *et al.*, 1993; Cho *et al.*, 1994), *SMAD4* (Takagi *et al.*, 1996; Thiagalingam *et al.*, 1996) and *MADR2* (Eppert *et al.*, 1996) are located there. *DCC* encodes a cell adhesion molecule, normally widely expressed on the colon mucosa (Hedric *et al.*, 1994). Its expression is reduced or absent in 70-75% of colon cancers (Vogelstein *et al.*, 1988; Fearon *et al.*, 1990; Itoh *et al.*, 1993; Cho *et al.*, 1994). Its role in colorectal carcinogenesis has however recently been questioned, since mutant mice lacking both alleles of *DCC* show no

abnormalities of intestinal biology (Fazeli *et al.*, 1997). Whether a neighbouring gene may in fact be the actual tumour suppresser gene on 18q remains to be determined. *SMAD4* and *MADR2* are known to be central players in the signal transduction pathway activated in response to the large family of TGFβ-like ligands. Frequent 8p loss detected in this study supports the hypothesis that other, as yet unidentified, oncosuppresser genes important in colorectal carcinogenesis are located on the short arm of chromosome 8 (Cunningham *et al.*, 1993; Fujiwara *et al.*, 1993; Kelemen *et al.*, 1994a, 1994b; Yaremko *et al.*, 1994; Farrington *et al.*, 1996). Possible candidates include *FEZ1* gene encoding Fez1 protein containing leucine-zipper region with similarity to the DNA-binding domain of the cAMP-responsive activating-transcription factor 5. *FEZ1* gene transcripts are undetectable in more than 60% of epithelial tumours and mutations in *FEZ1* have been found in oesophageal cancers and prostate cancer cell lines, suggesting that its inactivation may play a role in development of various human tumours (Ishii *et al.*, 1999). Other possible candidates include a gene frequently deleted in human liver cancer *DLC1* (*d*ynein *l*ight-*c*hain gene *1*; 8p21.3 - p22), *PRLTS* (*P*DGF-*r*eceptor β-*l*ike *t*umour *s*uppresser), *EXT1* and *EXTL3* [exostoses (multiple)-like 3] (Knuutila *et al.*, 1999). Deletions within the short arm of chromosome 1 in colorectal carcinomas have been previously reported (Leister *et al.*, 1990; Praml *et al.*, 1995; di Vinci *et al.*, 1996; Matsuzaki *et al.*, 1998). One of the three commonly deleted regions in chromosome 1 overlaps with the region to which *PLA2s* has been mapped. *PLA2s* encodes type II non-pancreatic phospholipase A2 (MacPhee *et al.*, 1995), one of the enzymes responsible for the production of arachidonic acid (a prostaglandin precursor). It is not clear however how loss of its function contributes to tumour development. In *min* mice, which are heterozygous for *APC* mutation and develop multiple bowel polyps in a similar manner to FAP patients (Moser *et al.*, 1990), a null mutation in one allele of *Mom1* (the mouse homologue of *PLA2s*) is associated with lower number of polyps. Region 1p36 contains another candidate for a tumour suppresser gene - *p73* which encodes a protein highly homologous to p53 (Kaghad *et al.*, 1997). However subsequent search for somatic mutations in *p73* gene in colorectal carcinomas revealed that such mutations are extremely rare (Han *et al.*, 1999). Human aflatoxin B-1 aldehyde reductase gene

located at 1p35-1p36 has also been suggested to play a role in colorectal carcinogenesis due to its presumptive involvement in detoxification of genotoxic and cytotoxic substances (Praml *et al.*, 1998). Other putative tumour suppressor genes located on the short arm of chromosome 1 include *ID3* (inhibitor of DNA binding 3; 1p36.13 - p36.12), *NB/NBS* (neuroblastoma suppressor; 1p36.13 - p36.11), *TNFR2* (tumour necrosis factor receptor 2; 1p36.3 - p36.2), *DAN* (differential-screening-selected gene aberrant in neuroblastoma; 1p36.13 - p36.11), *CDC2L1* (cell division cycle 2-like 1; 1p36) and *BRCD2* (breast cancer suppressor-2; 1p36) (Knuutila *et al.*, 1999) but their role in colorectal carcinogenesis is unclear. The search for specific tumour suppresser genes located on the short arm of chromosome 1 continues. Gain of chromosome material on 20q has been reported in various malignancies including colorectal cancer (Kallioniemi *et al.*, 1994; Iwabuchi *et al.*, 1995; Schlegel *et al.*, 1995; Reznikoff *et al.*, 1996; Larramendy *et al.*, 1997; Mahlamaki *et al.*, 1997) and the location of *STK15* on 20q13 makes it a likely target of this amplification. *STK15* (also known as *BTAK* and *aurora2*) encoding a centrosome-associated kinase is amplified and overexpressed in many human tumour cell types (Sen *et al.*, 1997; Bischoff *et al.*, 1998). It is involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells (Zhou *et al.*, 1998).

It is difficult to speculate on the significance of 8q and 13q duplications, since the regions of amplification include such large portions of the genome in which so far no genes of known importance in colorectal cancer are located.

The fact that there was an excellent consistency (92%) in the presence of chromosomal changes characteristic of RER- cancers (18q-, 8p-, 1p-, 20q+, 13q+, 8q+) between primary tumours and the corresponding xenografts and that other chromosomal changes were less frequently preserved indicates that these particular chromosomal amplifications or deletions gain tumour cells growth advantage and are therefore specifically selected for during tumour development.

4.4.3.2. Chromosomal abnormalities in RER+ sporadic colorectal cancers.

A different subset of chromosomal changes was found to be present most frequently in RER+ tumours. Although loss of the short arm of chromosome 1 appeared with similar frequency in both tumour groups, the frequent occurrence of 19del in RER+ tumours is a surprising finding and has not been previously reported. It is of particular interest now, since the Peutz-Jeghers gene has recently been mapped to the short arm of chromosome 19 (19p13.3) by linkage analysis (Hemminki *et al.*, 1997) and was subsequently found to encode serine threonine kinase *STK11* (Jenne *et al.*, 1998; Hemminki *et al.*, 1998). Data gathered to date indicate that LOH in this locus appears in 20%-50% of sporadic colorectal cancers (Resta *et al.*, 1998; Dong *et al.*, 1998). It is difficult to interpret the importance of the loss of chromosome 19 and the short arm of chromosome 1 in RER+ colorectal cancers. Our results showing frequent 19del in these tumours should be interpreted cautiously due to occasional aberrant results yielded by CGH for this particular chromosome (Kallioniemi *et al.*, 1994). However, the aberrant result usually represented gain rather then loss of chromosome 19. The small number of RER+ colorectal cancers included in this study further complicates the interpretation of the results. However, low consistency (57%) of the presence of these chromosomal changes between primary tumours and their xenografts suggests it is unlikely they confer a substantial positive growth advantage on cells. In view of this data, it can not be convincingly claimed that these abnormalities are specifically selected for in RER+ colorectal cancers.

4.4.4. Colorectal cancer xenografts are in general representative of the tumours of origin with regard to chromosomal abnormalities.

That more chromosomal abnormalities were detected in the xenografts compared to primary tumours can be partially explained by the fact that the test DNA isolated from primary tumours was consistently contaminated to some degree with normal DNA which decreases the CGH sensitivity. However, since the same error applied to both tumour groups the difference in the proportion of chromosomal abnormalities detected in the primary tumours when compared with their xenografts, which was greater in RER+ than in RER- cancers (58% and 45% respectively) could only be

explained by additional chromosomal changes appearing during the passage in the xenografts derived from RER- tumours. This difference indicates, although indirectly, the presence of an underlying mechanism of chromosomal instability in a proportion of RER- colorectal cancers.

The analysis of chromosomal abnormalities detected in the colorectal cancer xenografts established from samples taken from multiple sites showed that most of the chromosomal changes present in the primary tumour can be detected in the corresponding xenografts. Consistency in detecting the same chromosomal abnormalities in a primary tumour and in the corresponding xenograft reached 75% in RER- and 56% in RER+ tumours. This number was substantially higher in RER- cancers (92%) when only typical chromosomal changes were considered (18q-, 8p-, 1p-, 20q+, 13q+, 8q+). It does not appear be the case with RER+ xenografts, where consistency of the presence of specific changes (19del, 1p-) in primary tumours and in the xenografts was only 57%. Although a lower consistency was observed between RER+ cancers and their xenografts, chromosomal abnormalities are known to be infrequent in these tumours and are likely to be less important in their development. In this sense RER+ colorectal cancer xenografts are representative of their tumours of origin in as far as they show a small number of random chromosomal changes.

4.4.5. A proportion of RER- colorectal cancers are characterised by a high level of chromosomal instability.

Substantial differences found in the degree to which the CIN index increased between primary tumours and xenografts of RER- and RER+ cancers (74% and 34% respectively) reflects the high degree of underlying chromosomal instability in some RER- tumours. This instability is also well illustrated in a proportion of RER- cancers by differences in the total DNA content detected by flow cytometry analysis at different sites within the tumour and in the corresponding xenografts. However, it should be pointed out that tumours that were shown to be diploid by flow cytometry, did exhibit some chromosomal abnormalities when examined by CGH. One possible explanation is that these few chromosomal changes fell below the level of sensitivity of the flow cytometry as a method of detecting chromosomal copy number. It is also

important to note that aneuploidy is a state not a rate and though sometimes equated with chromosomal instability, aneuploidy could result from many factors other than a persistently elevated rate of chromosomal change. This could explain aneuploid DNA content detected by flow cytometry in some of the tumours showing few chromosomal changes by CGH, including one of the RER+ tumours. Alternatively, although one of the two mechanisms of genomic instability has been suggested to be sufficient for driving the neoplastic process both can coexist. The presence of MIN does not preclude the presence of CIN and vice versa (Lengauer *et al.*, 1997b). A recent study suggests, however, a good correlation between aneuploidy and chromosomal instability (Miyazaki *et al.*, 1999).

The results of the study strongly suggest that similar to MIN in RER+ cancers, CIN is a dynamic process which progresses with time. It further supports the hypothesis that CIN is a mechanism underlying tumorigenesis in a proportion of RER- colorectal cancers.

4.4.6. Molecular mechanisms underlying chromosomal instability.

While the mechanisms underlying microsatellite instability are known to involve the mismatch repair system (Peltomaki *et al.*, 1993; Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Papadopoulos *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996; Akiyama *et al.*, 1997; Miyaki *et al.*, 1997) and are relatively well understood, the molecular basis of the chromosomal instability present in the majority of malignancies is just beginning to be explored.

Defects in p53 function have been implicated as contributory factor in destabilisation of the genome. *In vitro* studies have shown that cells in culture often become grossly aneuploid at the same time that p53 is inactivated (Harvey *et al.*, 1993). Abnormalities of p53 have also been shown to precede aneuploid clonal divergence in colorectal cancer and are known to strongly predispose to chromosomal instability in many circumstances (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993; Deangelis *et al.*, 1993; Meling *et al.*, 1993; Bouffler *et al.*, 1995; Carder *et al.*, 1995; Donehower *et al.*, 1995; Gualberto *et al.*, 1998; Venkatachalam *et al.*, 1998). It is unlikely, however, that p53 is generally responsible for CIN, as several

cancer lines with *p53* mutations are diploid and chromosomally stable (Lengauer *et al.*, 1997b, Eshleman *et al.*, 1998a). Additionally, some studies indicate that aneuploidy appears early during tumorigenesis (Steinbeck *et al.*, 1994; Bardi *et al.*, 1997b, Bomme *et al.*, 1998), while *p53* mutations do not usually occur until later (Baker *et al.*, 1990; Auer *et al.*, 1994). These results indicate that, although p53 defects are unlikely to be a primary cause of chromosomal instability they are probably permissive to this process.

It is suspected that, in contrast to MIN where only a few genes are responsible for the phenotype there is a large number of genes which when altered can give rise to CIN. They include genes involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function and centrosome/microtubule formation and dynamics, as well as "checkpoint" genes that monitor the progression of the cell cycle (Lengauer *et al.*, 1998).

Altered expression of certain spindle checkpoint genes can result in aneuploidy. For example decreased expression of *hsMAD2* was observed in the T47D breast cancer cell line, which failed to undergo mitotic arrest after nocodazole (a mitotic spindle inhibitor) treatment suggesting that loss of *hsMAD2* function might lead to aberrant chromosome segregation and aneuploidy (Li and Benezra , 1996). In addition a small fraction of colorectal cancer cell lines that exhibit CIN have been shown to contain somatic mutations in mitotic checkpoint genes such as *hBUB1* and *hBUBR1* (Cahill *et al.*, 1998).

Several genes involved in DNA-damage checkpoints have also been implicated in contributing to the CIN phenotype, including *ataxia telangiectasia mutated* (ATM) (Rotman and Shiloh, 1998), the *ATM*-related gene *ATR* (Smith *et al.*, 1998), the *BRCA1* and *BRCA2* genes, which interact with the human *Rad51* homologue and *p53* (Lane, 1998). DNA-damage checkpoints prevent cells with DNA damage from entering mitosis, which could result in an inappropriate chromosome segregation due to sister chromatids being still connected by DNA or DNA-protein links (Lengauer *et al.*, 1998).

Another potential cause of CIN involves centrosomes, an abnormal number of which have been noticed in various human malignancies (Doxsey, 1998). Multipolar spindles

have often been observed in human cancers, but the molecular and genetic bases for the increased number of centrosomes have not yet been defined. Certain genes such as one encoding kinases aurora2/STK15 have however been implicated (Bischoff *et al.*, 1998; Zhou *et al.*, 1998).

Despite these clues, the molecular bases of CIN remains unknown in most human cancers. The fact that genetic defects in so many genes can lead to CIN might explain why this phenotype is so common. Accordingly, with so many genes involved, each one probably plays a role in a small proportion of cases (Lengauer *et al.*, 1998).

4.4.7. Molecular mechanisms that could lead to the development of a novel non-MIN, non-CIN phenotype in sporadic colorectal cancer.

It is likely that other than previously discussed mechanisms of genomic instability contribute to the development of non-CIN non-MIN tumours and there are a number of conceivable pathways that could result in the development of a colorectal carcinoma displaying neither of the two major genomic instability phenotypes. Recently a novel human mutator phenotype was described in the colon cancer cell line Vaco411, which increases the spontaneous mutation rate 10-100 fold over background (Eshleman *at al.*, 1998b). Transversion base substitutions were found to predominate and frameshifts, commonly seen in MMR defective cells, were not detected in this cell line. If Vaco411 was also lacking chromosomal instability its phenotype could resemble the phenotype of non-MIN non-CIN cancers detected in our series. Alternatively, an atypical MMR deficiency cannot be excluded, especially considering that MMR in bacteria requires at least ten individual components (Modrich, 1991) and that several components of the more complex human system are currently unknown (Kolodner, 1996; Modrich and Lahue, 1996). In this context, it is worth considering that approximately 15% of HNPCC tumours do not exhibit microsatellite instability (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Aaltonen *et al.*, 1994; Wu *et al.*, 1994) but may well harbour germline mutations in one of these undiscovered "caretakers" (Kinzler and Vogelstein, 1997). It is unlikely that these tumours harbour undetected classical microsatellite instability since the loci by which their RER status was established included one known to be extremely sensitive to

defects of mismatch repair (Hoang *et al.*, 1997). Other candidates include DNA repair systems involved in protecting the genome from genotoxic insult such as nucleotide excision repair and base excision repair. It would however be difficult to predict the phenotype resulting from defects in these genes as it would depend on the type of DNA lesions induced by environmental carcinogens. Finally, the disregulation of DNA methylation patterns in tumour cells can result in altered gene expression (Laird and Jaenisch, 1996; Jones and Gonzalgo, 1997; Laird, 1997) and could potentially drive tumorigenesis. Recently a novel hypermethylator phenotype was described in a subset of sporadic colorectal cancers (Toyota *et al.*, 1999). Through its ability to silence multiple genes simultaneously, CpG island methylator phenotype would be functionally equivalent to genetic instability, resulting in rapid accumulation of molecular alterations with a potential to accelerate the neoplastic process. Alternatively, non-MIN, non-CIN cancers might represent cases where carcinogenesis proceeds exclusively through selection of randomly occurring advantageous mutations and clonal expansion of tumour cells without acquiring a mutator phenotype. This model of tumorigenesis was proposed by Tomlinson *et al.* (1996) who used a mathematical model, based on colorectal cancer, to analyse the role of the mutation rate in the growth of sporadic tumours. This argues that selection without increased mutation rates is sufficient to explain the evolution of tumours. Further detailed studies of a larger series of colorectal cancers are required to determine the phenotypes of tumours displaying neither MIN nor CIN and to identify a driving force behind this neoplastic process.

4.5. Summary.

The most important finding of this study is the identification of a novel group of sporadic colorectal cancers which do not display instability of either chromosomes or microsatellites (called non-MIN, non-CIN cancers). These tumours do not show any striking differences in clinical and pathological features compared with RER- tumours exhibiting high levels of chromosomal instability but may harbour fewer abnormalities of p53 (see Chapter 5). The data also suggest that these cancers display a different pattern of clonal chromosomal abnormalities than high CIN RER- tumours, indicating that a different subset of tumour suppresser genes might be targeted in this tumour group. It is likely that non-MIN, non-CIN colorectal cancers represent a distinct entity in sporadic colorectal cancer and based on this data, their prevalence might be as high as 35% of RER- colorectal cancers or 25% of sporadic colorectal cancers in total. These tumours might harbour one or more novel mechanisms of genomic instability but alternatively may represent a group of sporadic colorectal cancers developing without an increased mutation rate through selection of advantageous mutations and clonal expansion.

The analysis of chromosome copy number changes in the RER- and RER+ groups of sporadic colorectal cancers confirmed previously reported differences in rates of chromosomal abnormalities occurring in these two cancer groups. Different patterns of chromosomal changes were found to occur in RER- and RER+ tumours. The specific pattern of chromosomal gains and losses identified in RER- sporadic colorectal cancers (20q+, 18q-, 13q+, 8p-, 1p- and 8q+) generally conforms with the results of previous cytogenetic and CGH studies. However, frequent loss of chromosome 19 in the RER+ group of tumours found in this study has not been previously reported. Differences in the rates at which chromosomal abnormalities occur, together with the different patterns of chromosomal changes appearing in RER- and RER+ colorectal cancers further support the hypothesis that pathways of carcinogenesis in these two groups of tumours are fundamentally different.

This study has identified the presence of genetic intratumoral heterogeneity in sporadic colorectal cancer. The analysis of colorectal cancer xenografts established

from samples collected from multiple sites from primary tumours showed that a xenograft established from a single sample is in general representative of its tumour of origin with regard to chromosomal abnormalities, despite the presence of genetic heterogeneity within primary tumours.

CHAPTER 5.

The role of p53 defects in RER- and RER+ sporadic colorectal cancers.

5.1. Introduction.

Defects in the *p53* tumour suppresser gene are the most common genetic changes found to date in human tumours (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Levine *et al.*, 1991 and 1994). Loss of p53 function occurs frequently in sporadic colorectal cancer and is found in up to 75% of cases (Baker *et al.*, 1990; Cunningham *et al.*, 1992; Vandenbroek *et al.*, 1993; Goh *et al.*, 1994). Mutations detected in *p53* in colorectal carcinomas are mostly missense mutations. They often result in the nuclear accumulation of abnormal protein with increased half-life, allowing detection by immunohistochemical methods. It has also been shown that 90% of all mutations in the *p53* gene are located in exons 5-8 (Levine *et al.*, 1991).

It is generally accepted that p53 plays a critical role in maintaining genomic integrity (Kastan *et al.*, 1991; Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Nelson and Kastan, 1994). Association of p53 defects with chromosomal instability is well documented (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993; Deangelis *et al.*, 1993; Meling *et al.*, 1993; Bouffler *et al.*, 1995; Carder *et al.*, 1995; Donehower *et al.*, 1995; Gualberto *et al.*, 1998; Venkatachalam *et al.*, 1998). There are also implications of p53 involvement in the regulation of *hMSH2* and it therefore has a possible role in mismatch repair (Scherer *et al.*, 1996).

Although published reports of the overall frequency of *p53* mutations in sporadic colorectal cancer are similar, conflicting data has appeared with regard to the frequency of *p53* mutations in RER- and RER+ colorectal carcinomas (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Kim *et al.*, 1994; Ilyas *et al.*, 1996; Cottu *et al.*, 1996; Remvikos *et al.*, 1997). Recent reports indicate that *p53* mutations are significantly less common in RER+ than RER- colorectal cancers (Cottu *et al.*, 1996; Eshleman *et al.*, 1998a). RER+ colorectal cancer cell lines with *p53* mutations have been shown to remain chromosomaly stable (Eshleman *et al.*, 1998a) indicating that *p53* mutation does not invariably induce chromosomal instability.

In order to determine the role of p53 in RER- and RER+ tumours p53 defects in the series of 22 sporadic colorectal cancers were investigated. Frequency of the loss of p53 function in both tumour groups was examined and its association with two types of genomic instability. The prevalence of p53 defects in previously identified non-MIN non-CIN group of sporadic colorectal cancer was also determined. Additionally by the immunohistochemical analysis of multiple sites within each tumour, p53 protein stabilisation patterns were analysed to estabilish whether one sample analysis is sufficient for determining p53 status by immunohistochemistry. In order to confirm that colorectal cancer xenografts are representative of the primary tumours they are established from with regard to p53 status, immunohistochemical analysis of all xenografts was carried out. In addition mutation analysis of selected xenografts corresponding to the primary tumour samples was performed.

Three different methods were used for determining p53 status. First immunohisochemistry analysis was carried out on all primary tumour and xenograft samples followed by mutation analysis of exons 5-8 of the representative samples and the corresponding xenografts. The loss of the short arm of chromosome 17 detected by CGH was also considered in the interpretation of the results.

5.2. Materials and Methods.

5.2.1. Immunohistochemical detection of stabilised **p53** *protein.*

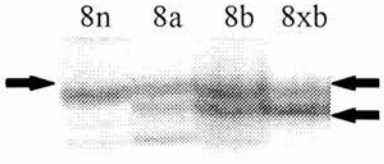
An immunohistochemical analysis was carried out using the DO-7 antibody (Dako Ltd, UK). This recognises an amino terminal epitope of the p53 protein and reacts with both wild type and mutant protein. A routine immunohistochemistry (IHC) protocol was followed as previously described (Purdie *et al.*, 1991), using Avidin-Biotinylated Horseradish Peroxidase Complex (ABComplex/HRP, Dako Ltd, UK) and 3,3'-diaminobenzidine (DAB) as a substrate on 3μm paraffin tissue sections. Detailed description of the protocol can be found in section 2.7. The slides were examined under 400x magnification on a light microscope and tumours were classified as p53 defective if more than 10% of nuclei showed intense positive staining.

5.2.2. Mutation analysis of the p53 gene.

Mutation analysis of the *p53* gene was performed on 15 tumours with low or negative immunohistochemical staining in order to exclude the possible presence of mutation not detectable by IHC. This analysis was also carried out on the corresponding xenografts. Previous studies of *p53* status at different tumour sites (Carder *et al.*, 1995), indicated that mutation in the *p53* gene, if present, occurs relatively early in tumour progression and therefore can be readily detected in any part of the tumour. For this reason, only one representative sample from each tumour was screened for the presence of mutation in the *p53* gene in the first instance. Exons 5-8, in which 90% of all mutations are located (Levine *et al.*, 1991), were amplified using the primers listed in Table 4 (see section 2.8) and details of PCR conditions can be found in section 2.8.

Single-stranded conformational polymorphism analyses (SSCP) with autoradiographic detection were undertaken as previously described (Carder *et al.*, 1995). Autoradiographs were assessed visually for shifts in electrophoretic mobility of amplified sequences compared to DNA from normal tissue of the same patient (see Figure 22).

Figure 22. *The result of SSCP analysis of ^{33}P-labelled PCR amplified exon 5 of p53 gene in primary tumour samples 8a and 8b and in the xenograft 8xb. The autoradiograph shows an additional band in tumour samples and in the xenograft compared to normal control, indicative of the presence of a mutation in this exon.*



5.2.3. Analysis of 17p loss detected by CGH.

Additionally a possible effect of the loss of the short arm of chromosome 17, where the *p53* gene is located, on p53 function was considered. Previously gathered CGH data was used.

5.3. Results.

5.3.1. **p53** *immunostaining.*

Within the group of 17 RER- colorectal cancers seven showed positive staining (strong nuclear staining present in more than 10% of cells), a further two tumours showed some degree of immunohistochemical reaction, which was not sufficient to classify them as positive (weak staining or staining present in less than 10% of cells). The remaining eight tumours showed no reaction and were classified as negative. Among RER+ cancers three showed some staining but none classified as positive. In the remaining two no staining was observed.

Based on these results 7 out of 17 RER- and none of the RER+ cancers were classified as p53 defective (see Tables 19 and 20). Substantial heterogeneity of intensity and patterns of staining within the tumours was observed. It applied to almost every single sample analysed, where different intensity and patterns of staining could be observed within one section. This, however, did not affect the overall result and the final scores for each site within the tumour were consistent. The results indicate that despite substantial intratumoral heterogeneity regarding intensity and pattern of immunohistochemical staining, a single sample analysis is sufficient for determining stabilisation of p53 protein in sporadic colorectal cancer.

The analysis of the staining patterns in the primary tumours and the corresponding xenografts showed very few discrepancies indicating that the xenografts are, in general, representative of the colorectal cancers they are derived from (see Tables 21 and 22). Inconsistencies were observed in xenografts established from tumour No 22 which showed little or no staining but was established from a p53 positive RER- primary tumour. Minor discrepancies were noticed in one RER+ tumour where some degree of staining was observed in the samples taken from the primary tumour and no

staining at all was present in the xenografts (see Tables 21 and 22). This, however, did not affect the score since both primary tumour samples and the xenografts were classified as negative.

5.3.2. Mutation analysis of the p53 gene.

Mutation analyses were carried out on representative samples from 10 RER- and all RER+ tumours. Out of 10 RER- tumours three showed the presence of a mutation, two in exon 5 and one in exon 6. Only one mutation, located in exon 5, was observed in the five RER+ tumours analysed.

In two cases where samples from multiple sites were analysed, (case No 19) and one RER+ tumour (case No 12) inconsistencies between different tumour sites were noticed. In both cases a mutation in the *p53* gene was detected in all but one of the multiple samples examined (see Figures 23 and 24). This can not be explained by a low concentration of tumour DNA in the analysed sample since in the case of the RER+ tumour it was sufficient to detect shifts in lengths of microsatellite sequences and in the case of sample 19a it did not affect the CGH analysis where chromosome copy number changes could still be detected. Therefore, these cases are most likely to represent genuine differences in p53 status within examined tumours.

The mutation analyses of xenografts showed that generally the results were consistent with those of the corresponding primary tumours (see Tables 21 and 22). In the case of the RER- tumour (case No 5), no mutation was observed in exons 5-8 but LOH at the *p53* locus was noticed in all three xenografts derived from this tumour (see Figure 25). These results are not necesserily inconsistent with the results obtained for the primary tumour, since presence of LOH in primary tumours is difficult to assess due to contamination with normal DNA. In one case (No 12) where one out of four samples collected from the primary tumour (12d) did not show the mutation in exon 5, the corresponding xenograft did (see Figure 24). However all other three samples collected from this tumour showed presence of a mutation in exon 5 and this mutation was detected in the corresponding xenografts.

Figure 23. *SSCP analysis of p53 exon 6.*
The sequence was PCR amplified and labelled with ^{33}PdATP. Mutation in exon 6 of the p53 gene was detected in two out three samples collected from this RER- tumour (case No 19). Xenografts established from the two samples with the p53 mutation showed presence of p53 mutation in the same exon. The origin of the xenograft 19xa is disputable and this case is discussed in detail in Chapter 6.

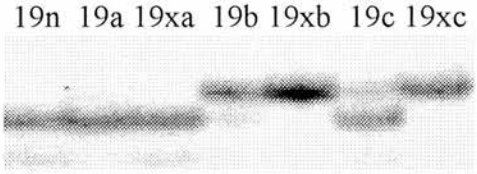


Figure 24. *SSCP analysis of p53 exon 5.*
The sequence was PCR amplified and labelled with ^{33}PdATP. P53 mutation was detected in all but one sample collected from this RER+ tumour (case No 12, sample 12d). This mutation was, however detected in the corresponding xenograft (12xd).

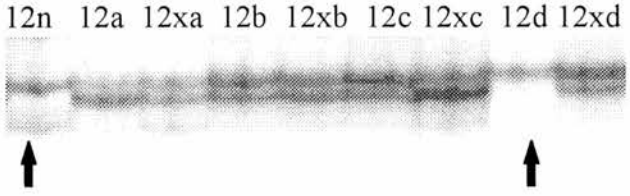
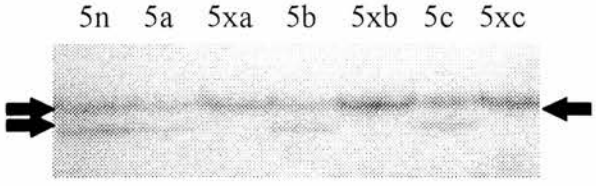


Figure 25. *SSCP analysis of p53 exon 6.*
The sequence was PCR amplified and labelled with ^{33}PdATP. In case No 5 (a RER- cancer), no mutation was detected in exons 5-8 but due to the presence of polymorphism in exon 6, there was evidence of LOH in p53 locus in the xenografts. It is difficult to assess LOH in the primary tumour due to contamination with normal DNA



5.3.3. 17p loss as detected by CGH.

The loss of the short arm of chromosome 17 was considered in determining p53 status, and the results are included in the Tables 19 and 20. It is difficult to predict significance of 17p loss in cases where no p53 protein stabilisation is seen and there is no evidence of *p53* mutation. The significance of LOH in *p53* locus without accompanying mutation in the other allele has not as yet been determined and in at least a proportion of tumours 17p loss might result from underlying chromosomal instability. However the dominant negative effect of *p53* mutation, inactivating the protein function has been suggested (Hollstein *et al.*, 1996). An oncogenic form of *p53*, with a missense mutation which confers a dominant gain-of-function phenotype disrupting spindle checkpoint control leading to genomic instability has also been described (Gualberto *et al.*, 1998).This is why tumours which showed 17p loss but neither p53 protein stabilisation nor the presence of a mutation in the exons examined, were treated as p53 proficient for the purpose of the statistical analysis. Cases where a mutation in *p53* gene was detected despite negative IHC were treated as p53 defective.

Table 19. *p53 status, DNA ploidy and the number of chromosomal changes detected by CGH in RER+ colorectal cancers.*

Sample ID	p53 IHC	p53 mutation analysis ex. 5-8	17p loss by CGH	Overall p53 status	Ploidy	DNA index	Total number of chromosomal changes by CGH	CIN index
3a	n	-	-	functional	diploid		0	
3b	n	-	-	functional	diploid		0	4
3c	n	-	-	functional	diploid		1	
3d	n	-	-	functional	diploid		2	
12a	n*	+(ex5)	-	defective	diploid		9	
12b	n*	+(ex5)	-	defective	diploid		7	23
12c	n*	+(ex5)	-	defective	diploid		2	
12d	n*	-	+	?	diploid		9	
17a	n*	-	-	functional	diploid		5	
17b	n*		-	functional	diploid		5	12
17c	n*		+	p.functional	diploid		7	
17d	n*		+	p.functional	diploid		8	
18a	n*	-	-	functional	near tetraploid	1.93	9	
18b	n*		-	functional	near tetraploid	1.95	4	23
18c	n*		-	functional	diploid		10	
18d	n*		-	functional	near tetraploid	1.87	2	
20a	n	-	-	functional	diploid		7	
20b	n		-	functional	diploid		1	10
20c	n		-	functional	diploid		4	
20d	n		-	functional	diploid		2	

Table 20. *p53 status, DNA ploidy and the number of chromosomal changes detected by CGH in RER- colorectal cancers.*

Sample ID	p53 IHC	p53 mutation analysis ex. 5-8	17p loss by CGH	Overall p53 status	Ploidy	DNA index	Total number of chromosomal changes by CGH	CIN index
1a	n	-	-	functional	near tetraploid	1.82	8	
1b	n		+	p.functional	near tetraploid	1.84	14	37
1c	n		+	p.functional	near tetraploid	1.84	20	
1d	n		-	functional	near tetraploid	1.82	6	
2a	p		-	defective	near diploid	1.1	12	
2b	p		-	defective	near tetraploid	2.2	28	47
2c	p		-	defective	n.dipl./n.tetrapl.	1.2/2.2	8	
2d	p		-	defective	near diploid	1.1	16	
4a	n*	+(ex5)	-	defective	aneuploid	1.54	13	
4b	n*		-	defective	aneuploid	1.6	11	27
4c	n*		-	defective	aneuploid	1.59	10	
4d	n*		-	defective	aneuploid	1.58	9	
5a	n	-*	-	p.functional	aneuploid	1.65	14	
5b	n	-*	-	p.functional	aneuploid	1.65	0	22
5c	n	-*	-	p.functional	aneuploid	1.58	2	
5d	n		-	p.functional	aneuploid	1.63	6	
6a	p		-	defective	aneuploid	1.66	10	
6b	p		-	defective	aneuploid	1.68	13	30
6c	p		-	defective	aneuploid	1.72	11	
6d	p		-	defective	aneuploid	1.68	9	
7a	p		-	defective	near tetraploid	1.81	0	
7b	p		-	defective	aneuploid	1.76	7	9
7c	p		-	defective	near tetraploid	1.85	0	
8a	n	+(ex5)	-	defective	aneuploid	1.68	1	
8b	n	+(ex5)	+	defective	aneuploid	1.73	10	26
8c	n		+	defective	aneuploid	1.76	8	
8d	n		-	defective	aneuploid	1.65	9	
9a	p		-	defective	near tetraploid	1.87	1	
9b	p		-	defective	near tetraploid	1.92	17	29
9c	p		-	defective	near tetraploid	1.96	6	
9d	p		-	defective	near tetraploid	2.04	10	
9a'	p		-	defective	diploid		8	13
9a'	p		-	defective	diploid		1	
10a	n	-	-	functional	diploid		2	
10b	n		-	functional	diploid		8	17
10c	n		-	functional	diploid		4	
11a	p		+	defective	aneuploid	1.43	17	
11b	p		+	defective	near tetraploid	2.05	11	48
11c	p		+	defective	aneuploid	1.45	25	
11d	p		-	defective	aneuplod	1.36	17	
13a	n	-	-	functional	diploid		5	10
13b	n*	-	-	p.functional	diploid		2	
14a	n	-	+	p.functional	near diploid	1.1	16	22
14b	n	-	-	functional	diploid		12	
15a	n	-	+	p.functional	aneuploid	1.4	3	13
15b	n	-	+	p.functional	diploid		10	
16a	n	-	+	p.functional	diploid		6	12
16b	n	-	-	functional	diploid		6	
19a	n	-	-	?	aneuploid	1.54	5	
19b	n	+(ex6)	+	defective	aneuploid	1.54	15	24
19c	n	+(ex6)	+	defective	aneuploid	1.52	17	
22a	p	+(ex5)	-	defective	aneuploid	1.32/2.43	7	
22b	p	+(ex5)	-	defective	aneuploid	1.32/2.3	3	23
22c	p	+(ex5)	+	defective	aneuploid	1.4/2.3	14	

Table 21. *p53 status, DNA ploidy and the number of chromosomal changes detected by CGH in RER- colorectal cancers and in the corresponding xenografts.*

Sample ID	p53 IHC		p53 mutation analysis ex. 5-8		17p loss by CGH		p53 status		Ploidy		DNA index		Total number of chrom. changes	
	p	x	p	x	p	x	p	x	p	x	p	x	p	x
1a/1xa	n	n	-	-	-	-	functional	functional	n.tetrapl.	n.tetrapl.	1.82	1.85	8	31
1b/1xb	n	n			-	-	functional	functional	n.tetrapl.	n.tetrapl.	1.84	1.89	14	32
1c/1xc	n	n			-	-	functional	functional	n.tetrapl.	n.tetrapl.	1.84	1.80	20	30
1d/1xd	n	n			-	-	functional	functional	n.tetrapl.	n.tetrapl.	1.82	1.84	6	16
4b/4xb	n*	n*	+(ex5)	+(ex5)	-	+	defective	defective	aneuploid	n.tetrapl.	1.6	1.94	11	20
5a/5xa	n	n	-*	al.loss	-	+	p.functional	p.functional	aneuploid	aneuploid	1.65	1.75	14	22
5b/5xb	n	n	-*	al.loss	-	-	p.functional	p.functional	aneuploid	aneuploid	1.65	1.64	0	9
5c/5xc	n	n	-*	al.loss	-	-	p.functional	p.functional	aneuoloid	aneuploid	1.58	1.63	2	20
8b/8xb	n	n*	+(ex5)	+(ex5)	+	-	defective	defective	aneuploid	aneuploid	1.73	1.75	10	24
19b/19xb	n	n	+(ex6)	+(ex6)	+	-	defective	defective	aneuploid	aneuploid	1.54	1.62	15	26
19c/19xc	n	n	+(ex6)	+(ex6)	+	-	defective	defective	aneuploid	aneuploid	1.52	1.63	17	24
22a/22xa	p	n*	+(ex5)	+(ex5)	-	-	defective	defective	aneuploid	aneuploid	1.32	1.34	7	29
22b/22xb	p	n	+(ex5)	+(ex5)	-	+	defective	defective	aneuploid	n.tetrapl.	1.32	1.8	3	23
22c/22xc	p	n*	+(ex5)	+(ex5)	+	-	defective	defective	aneuploid	aneuploid	1.4	1.42	14	25

Table 22. *p53 status, DNA ploidy and the number of chromosomal changes detected by CGH in RER+ colorectal cancers and in the corresponding xenografts.*

Sample ID	p53 IHC		p53 mutation analysis ex. 5-8		17p loss by CGH		p53 status		Ploidy		DNA index		Total number of chrom. changes	
	p	x	p	x	p	x	p	x	p	x	p	x	p	x
12a/12xa	n*	n	+(ex5)	+(ex5)	-	+	defective	defective	diploid	diploid			9	13
12b/12xb	n*	n*	+(ex5)	+(ex5)	-	+	defective	defective	diploid	diploid			7	7
12c/12xc	n*	n*	+(ex5)	+(ex5)	-	-	defective	defective	diploid	diploid			2	8
12d/12xd	n*	n*	-	+(ex5)	+	+	?	defective	diploid	diploid			9	12
18a/18xa	n*	n	-	-	-	-	functional	functional	n.tetrapl.	n.tetrapl.	1.93	1.83	10	20
18b/18xb	n*	n			-		functional	functional	n.tetrapl.	n.tetrapl.	1.95	1.83	4	
18c/18xc	n*	n			-	-	functional	functional	diploid	diploid			9	6
18d/18xd	n*	n			-		functional	functional	n.tetrapl.	n.tetrapl.	1.87	1.98	2	
20a/20xa	n	n	-	-	-	-	functional	functional	diploid	diploid			7	11
20b/20xb	n	n			-	-	functional	functional	diploid	diploid			4	9
20c/20xc	n	n			-	-	functional	functional	diploid	diploid			2	7

Key to Tables 19-22:

p, primary tumour
x, xenograft
p53 IHC - p53 immunohistochemistry results by DO7:
n, negative (no staining observed)
n*, negative (weak staining observed or staining present in 10% of the cells or less)
p, positive (strong staining in more than 10% of cells
p53 mutation analysis:
-, no mutation found in exons 5-8 by SSCP
+, mutation found by SSCP
-*, no mutation found, but LOH was noticed by SSCP
al.loss, allele loss by SSCP
17p loss:
-, not detected by CGH
+, detected by CGH
p53 status:
p.functional, probably functional
p.defective, probably defective
?, difficult to interpret the results
Ploidy:
n.tetrapl., near tetraploid (samples were classified near tetraploid if the tumour cells' peak fluorescence value was within 10% tetraploid peak fluorescence value, but for statistical purposes were treated as aneuploid)
DNA index:
The DNA index was calculated as the ratio of peak position values of the aneuploid and diploid peaks.

5.4. Discussion.

The overall frequency of p53 defects (protein stabilisation detected by IHC or presence of a mutation detected by SSCP) in our series of sporadic colorectal cancers was 50%. These results are generally in concordance with previously published data (Rodrigues *et al.*, 1990; Vandenbroek *et al.*, 1993; Goh *et al.*, 1994). Although p53 defects were more frequently found in RER- cancers compared to RER+ cancers (in 10 out of 17 and in 1 out of 5 respectively) this difference did not appear statistically significant (Fisher exact test p=0.31). However, analysis of the frequency of p53 defects in tumours with high (CIN>18) and low (CIN<18) chromosomal instability indices (see section 4.3.5) showed that p53 is more often defective in tumours with high level of chromosomal abnormalities regardless of their RER status and this difference is statistically significant (one-tailed Fisher exact test p=0.04). These results are in general consistent with the findings of Eshleman *et al.* (1998a), showing that RER+ colorectal cancer cell lines with *p53* mutations remain chromosomally stable. This indicates that chromosome number and structure in RER+ colon cancers is independent of whether p53 is mutant or wild-type. However in this series one of the two RER+ tumours showing a high level of chromosomal instability did harbour a *p53* mutation. Additionally most of the RER- cancers expressing high levels of chromosomal instability (8 out of 11) were shown to have defective p53 unlike RER- tumours with low CIN index where only two out of six were found to be p53 defective. This difference however was not statistically significant. Thus both this study and that of Eshleman *et al.* (1998a) indicate that *p53* mutation does not invariably induce chromosomal instability, although it is strongly associated with it.

It is not clear exactly how p53 contributes to the maintenance of a stable genome. It has been suggested to play a role in the G1-S checkpoint (Kastan *et al.*, 1991 and 1992; Kuerbitz *et al.*, 1992) by delaying cell cycle progression in order to allow DNA repair and thus inhibiting replication of cells with damaged DNA. It is also implicated in the G2-M 'spindle-surveillance' checkpoint which prevents the survival of cells with aneuploid or polyploid DNA content (Gottlieb and Oren, 1996). Although evidence indicates that p53 defects in themselves are not sufficient to trigger genomic instability in colorectal cancer, loss of normal p53 function followed by loss

of cell cycle control permits the rapid accumulation of genetic changes necessary for tumour development. Its exact role however in chromosomally unstable tumours still remains to be determined.

The fact that *p53* mutations are quite infrequent in RER+ colorectal cancers indicates that loss of its function is not prerequisite in the development of this type of genomic instability. Considering that p53 is directly involved in recognition of insertion/deletion mismatches (Lee *et al.*, 1995) and binds to the promoter region of *hMSH2* (Scherer *et al.*, 1996) implies its role in mismatch repair. It is not as yet clear however whether loss of p53 function facilitates accumulation of point mutations in MMR deficient cells or not. A number of *in vitro* studies investigating the effect of p53 on the spontaneous mutation frequency have indicated that p53-null cells do not bear an increased mutation load (Nishino *et al.*, 1995; Sands *et al.*, 1995; Clarke *et al.*, 1997). There is however evidence that p53 status might have significant effects on the mutation frequency following exposure to DNA damaging agents (Corbet *et al.*, 1999). Further studies are necessary to clarify the p53 role in both types of genomic instability.

Analyses of the frequency of p53 defects in the previously identified non-MIN, non-CIN group of sporadic colorectal cancers showed that p53 is not often targeted in this group of tumours. Defects in p53 were found in 2 out of 6 (33%) low CIN, RER- cancers compared to 8 out of 11 (73%) in high CIN, RER- tumours. These findings, together with the specific chromosomal abnormalities identified in this group of tumours (see section 4.3.6) indicate that a different subset of tumour suppresser genes might be targeted in non-MIN, non-CIN cancers.

The immunohistochemical analysis of multiple sites within the tumours proved that despite the presence of substantial intratumoral heterogeneity regarding the pattern and intensity of staining, a single sample analysis is sufficient for detecting stabilisation of p53 protein in sporadic colorectal cancer. Additionally the results indicate that weak staining or staining present in less than 10% of cells is not always associated with *p53* mutation, although a possibility of a mutation being present in an exon other than 5,6,7 or 8 can not be excluded. Mutations in *p53* were detected in 2 out of 5 colorectal cancers showing some degree of immunohistochemical reaction

which was not sufficient to be classified as positive. Two out of 10 tumours that showed no p53 protein stabilisation at all were proved to carry *p53* mutation and one showed an allele loss in *p53* locus. These results are in keeping with data published previously regarding the consistency between p53 IHC staining and mutations in the *p53* gene (Cripps *et al.*, 1994; Dix *et al.*, 1994; Bosari *et al.*, 1995; Costa *et al.*, 1995).

Due to some discordance between p53 protein stabilisation detected by IHC and the presence of a mutation in the *p53* gene, determination of p53 status in sporadic colorectal cancers requires the use of additional techniques (ie.SSCP) to detect *p53* mutations and the employment of stringent criteria in the interpretation of immunohistochemistry results.

Discrepancies between different sites within the tumour regarding the presence of *p53* mutation were found in two cases where multiple tumour samples were analysed. These discrepancies, however, are rare and in keeping with previous findings indicating that *p53* mutations can occur as a late event in colorectal carcinogenesis (Baker *et al.*, 1990; Auer *et al.*, 1994; Ilyas *et al.*, 1996).

Although loss of the chromosome material on the short arm of chromosome 17 has been shown to frequently accompany the mutation in the *p53* gene (Baker *et al.*, 1990; Cunningham *et al.*, 1992) in our series of sporadic colorectal cancers it was found in exactly the same proportion of p53 defective tumours and cancers with normal p53 function (5 out 11 tumours in each case). Thus, although previous studies suggested that additional loss of the wild-type allele accompanying *p53* mutation might gain tumour cells further growth advantage, the distribution of 17p loss in the series of sporadic colorectal cancers examined in this study is not supportive of this hypothesis. In at least a proportion of tumours 17p loss might result from an underlying chromosomal instability.

Comparison of the p53 status in the primary tumours and in the corresponding xenografts showed minor discrepancies mostly regarding staining patterns and in a few cases 17p loss. However, these discrepancies did not influence the final classification of primary tumours and xenografts as p53 deficient or proficient. These results indicate that colorectal cancer xenografts are representative of the primary

tumours they are established from with regard to their p53 status. They therefore represent a valuable tool in investigating the response of p53 proficient and deficient colorectal cancer cells to various therapeutic agents.

5.5. Summary.

The study showed that p53 defects appear more frequently in tumours exhibiting high level of chromosomal instability, regardless of their RER status and that these defects are significantly less common in colorectal cancers with low levels of chromosomal instability. The results also indicate that p53 is not a frequent target in non-MIN, non-CIN cancers. This study showed that single sample analysis is sufficient for detecting stabilisation of p53 protein but additional tests are usually required for adequate determining of p53 status in sporadic colorectal cancers. Satus of p53 in the primary tumour was shown to remain unchanged in corresponding xenografts.

CHAPTER 6.

Investigation of the origin of xenografts 3xb and 19xa.

6.1. Introduction.

Previous studies have demonstrated that the RER phenotype of the primary tumour is invariably preserved in colorectal cancer xenografts (Curtis, 1998). The results of this study are generally in concordance with these data. However, in two cases discrepancy between the RER status of primary tumours and corresponding xenografts was observed. These two cases could represent a rare but extremely important phenomenon of alteration of the RER phenotype of tumour cells during passage in SCID mice. Although sample mix-up was not suspected, in both cases where discordance of the RER phenotype was observed, DNA fingerprinting analysis was carried out to establish whether the xenograft's DNA matched the DNA of the tumour of origin and normal DNA from the patient from whom the tumour was obtained. In addition in one case the histogenesis of tumour cells in the xenograft was investigated.

It was important to determine whether the RER+ phenotype can spontaneously appear or disappear in the process of establishing colorectal cancer xenografts in SCID mice. Mismatch repair deficient cells have been shown to confer sensitivity to chloroethylating agents (Liu *et al.*, 1996) but resistance to temozolomide, alkylating agents and cisplatin (Branch *et al.*, 1993 and 1995; Kat *et al.*, 1993; de Wind *et al.*, 1995; Aebi *et al.*, 1996; Drummond *et al.*, 1996a and 1996b; Fink *et al.*, 1996; Liu *et al.*, 1996; Mello *et al.*, 1996). Presence or absence of RER+ phenotype in colorectal cancer xenografts indicates deficiency or proficiency of MMR system in tumour cells. This may substantially influence their response to various therapeutic agents. It is therefore prerequisite for proper assessment of genotype-dependent treatment response, carried out on colorectal cancer xenografts, to establish beyond doubt that they are in all cases representative of their tumour of origin with regard to the RER status.

6.2. Materials and methods.

The probe 29C1 was chosen for DNA fingerprinting. It recognises a highly polymorphic region of human telomeric DNA located in the pairing regions of the short arms of the sex chromosomes (Cooke *et al.*, 1985) which is not susceptible to mismatch repair defects. The analysis was carried out according to the protocol described in section 2.9 on the xenografts 3xb and 19xa. In each case the DNA extracted from the xenograft, the presumed sample of origin and normal mucosa from the patient from whom the tumour was obtained, was analysed for comparison. In one case, 3xb, DNA fingerprinting using microsatellite sequences was carried out. The analysis was performed by Mr Peter Han from the Police Forensic Science Laboratory using a set of seven microsatellite markers. The choice of microsatellites as markers in this particular case was appropriate since the xenograft 3xb proved to contain stable microsatellites and could be matched with patient's normal DNA and their tumour which also contained some normal DNA in contaminating stroma.

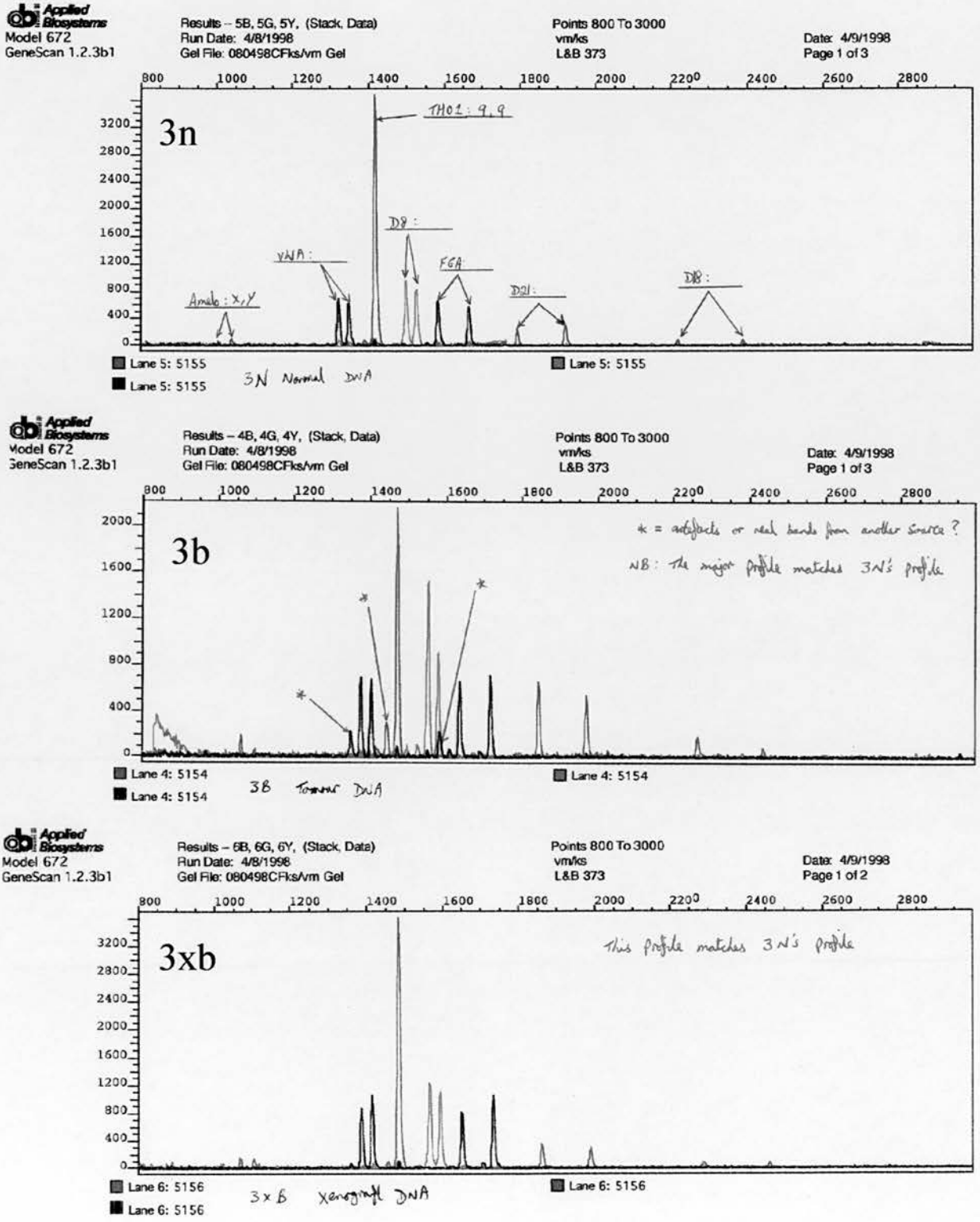
After confirming that the xenograft 3xb was established from the tissue obtained from the same patient from whom normal tissue (3n) and a sample of the primary tumour (3b) was collected, it became necessary to establish the histogenesis of tumour cells in the xenograft, since histological patterns of the primary tumour and the investigated xenografts differed substantially. This was done by immunohistochemistry using a panel of antibodies. Three μm paraffin sections of the xenograft 3xb were examined for the presence of cytokeratin (CAM 5.2), common leucocytic antigen (CD45), B-cells antigen (CD20), pan-T-cell marker (CD3) and Ebstein-Barr virus (EBV).

6.3. Results.

6.3.1. The origin of xenograft 3xb.

The xenograft 3xb was established from an RER+ colorectal cancer and despite implanting multiple samples collected from four different sites, this was the only xenograft successfully established from this tumour. Unlike sample 3b from the corresponding primary tumour, which showed shifts in three out of four analysed loci, no shifts were observed in the corresponding xenograft 3xb and this xenograft was classified as RER-. The results of DNA fingerprinting analysis, using the 29C1 probe, were inconclusive (see Figure 26a). The xenograft's DNA showed the presence of two bands present in the primary tumour and in the normal tissue but it lacked the third band and showed an additional two bands not found either in the primary tumour or the normal tissue. For this reason the samples were analysed by the Police Forensic Science Laboratory using a set of seven microsatellite markers. This analysis showed that the normal (3n), tumour (3b) and xenograft (3xb) DNA matched each other, which confirmed that tumour No 3 was the tumour of origin for xenograft 3xb (see Figure 27). This indicated that the RER+ phenotype was not preserved in this colorectal cancer xenograft. However the study of histological patterns of the primary tumour and the xenograft revealed that, while the tumour of origin was a moderately differentiated adenocarcinoma, the corresponding xenograft represented an anaplastic tumour in which the epithelial origin of tumour cells was not apparent. Immunohistochemical analysis with a panel of antibodies confirmed that tumour cells in the xenograft 3xb carried white cell markers (there was a strong immunohistochemical reaction with antibodies against common leukocytic antigen CD45 and B-cells marker CD20) but did not show the presence of cytokeratine filaments. These results facilitated the diagnosis of the xenograft 3xb as a large cell B-cell lymphoma. Additionally the 3xb cells showed positive reaction with antibodies against EBV. The significance of this finding is discussed later in this chapter (section 6.4), but due to the nonepithelial nature of the xenograft 3xb, it was not used in the study of chromosomal abnormalities.

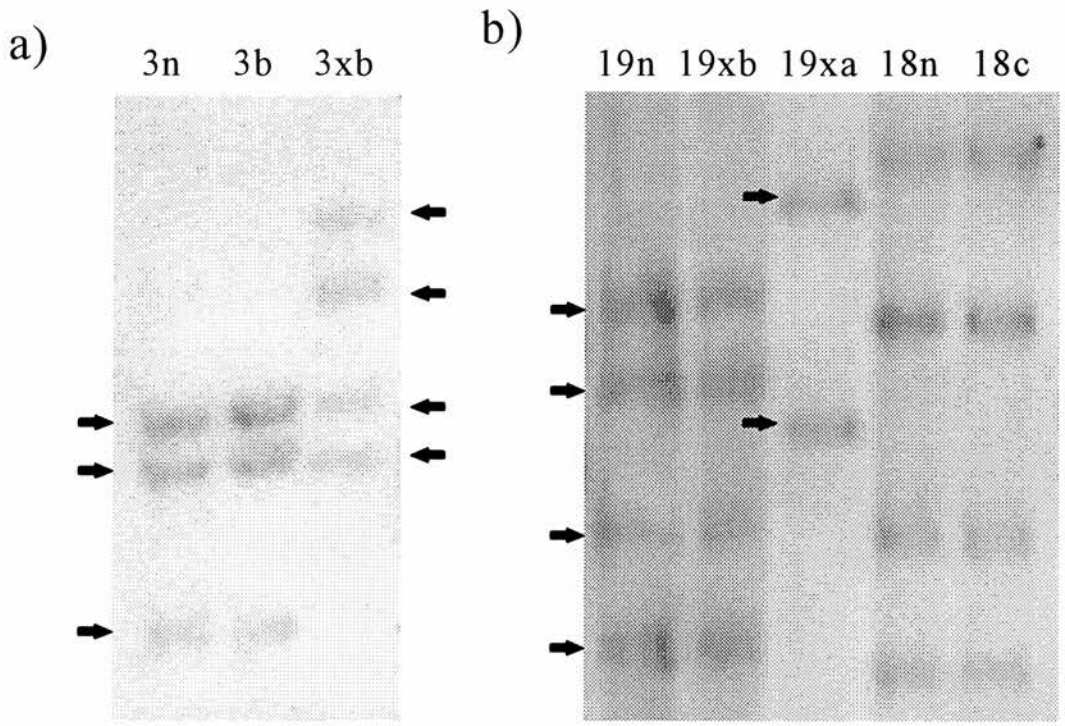
Figure 27. *The results of DNA fingerprinting using a set of seven microsatellite loci carried out in the Police Forensic Science Laboratory by Mr Peter Han. This shows that the microsatellite profile of samples 3n(normal DNA), 3b (tumour DNA) and 3xb (xenograft's DNA) match, confirming that tumour No3 is a tumour of origin for the xenograft 3xb.*



6.3.2. The origin of xenograft 19xa.

The analysis of microsatellites in xenograft 19xa showed shifts at all four loci examined, contrary to the primary tumour sample (19a) where microsatellites were shown to be stable. If sample 19a was proved to be the sample of origin for the xenograft 19xa it would indicate that the RER+ phenotype can spontaneously occur during colorectal cancer xenograft establishment or passage. DNA fingerprinting using the 29C1 probe of normal DNA (19n), DNA from another xenograft established from this primary tumour (19xb) and DNA from xenograft 19xa showed however that only samples 19n (normal tissue) and 19xb (another xenograft established from this primary tumour) matched each other. The sample 19xa showed an entirely different band pattern (see Figure 26b). The results indicated strongly that tumour No19 was not the tumour of origin for xenograft 19xa. For this reason 19xa was not included in any of the calculations relating to chromosomal abnormalities.

Figure 26. *Autoradiograph of Southern blot hybridised with ^{32}P-labelled 29C probe after DNA digested with EcoRI and electrophoresis.*



DNA was digested with EcoRI and electrophoresis was carried out overnight against 1kb ladder marker. The marker is not shown since determination of band sizes is not necessary for the interpretation of the results. Band patterns were compared between samples (bands are indicated by black arrows) to determine their origin.

a) Normal (3n) and primary tumour (3b) DNA match each other but the xenograft (3xb) shows presence of two extra bands not detected in the first two samples and lacks one band present in the primary tumour and the normal tissue. The results are inconclusive.

b) DNA from the xenograft 19xa does not match normal DNA (19n) and DNA from xenograft 19xb which was established from the same primary tumour as 19xa. Samples 18n (normal tissue) and 18c (RER+ primary tumour tissue) are shown to illustrate that the sequence chosen for this analysis is not affected by defects of mismatch repair and can provide conclusive results in such cases.

6.4. Discussion.

This analysis aimed to establish beyond doubt the origin of two colorectal cancer xenografts where discrepancy in the RER status with the primary tumour was observed.

Although in each case the attempt was made to establish colorectal cancer xenografts in SCID mice in the case of xenografts 3xb cancer cells failed to grow in the mouse environment. Instead, the xenograft consisted of transformed human B lymphocytes which exhibited EBV positivity and were proved to derive from the same patient from whom the primary colorectal cancer was obtained. SCID mice lack functional B and T lymphocytes, and therefore do not reject foreign tissue. For this reason they are widely used to grow and study various human malignancies including colorectal cancers and B-cell lymphomas. The Epstein-Barr virus can be found in most humans as a lifelong latent infection established in host B cells after a primary viral encounter (Haque *et al.*, 1996; Pisani *et al.*, 1997; Crowcroft *et al.*, 1998). In immunosuppressed individuals, such as post-transplant patients, the presence of EBV-infected B cells may lead to lymphoproliferative disease (Wood *et al.*, 1996; Lucas *et al.*, 1997; Quintanilla-Martinez *et al.*, 1998; Nalesnik, 1998). Injection of human peripherial blood lymphocytes from EBV-positive donors into SCID mice induces human lymphoproliferative disease in the recipient closely resembling that of human post transplant patients (Mosier *et al.*, 1990; Rowe *et al.*, 1990; Garnier *et al.*, 1993; Murphy *et al.*, 1995; Fuzzati-Armentero and Duchosal, 1998). The diagnosis of primary lymphoproliferative disease was not confirmed in the patient from whom the primary colorectal cancer No3 was obtained. The fact that xenograft 3xb consisted of transformed human B-lymphocytes can be explained by EBV-infected B-cells present in the tumour inflammatory infiltrate undergoing malignant transformation and expansion in the immunodeficient environment after implantation of tumour tissue in SCID mouse. Expanding transformed lymphocytes possibly suppressed the growth of colorectal cancer cells. Interestingly, this "new" tumour did not exhibit microsatellite instability, indicating that multiple malignancies arising from one individual's cells need not necessarily follow the same pathway of

tumourigenesis. This example illustrates the necessity for thorough investigation in any cases where the RER+ phenotype of the primary tumour does not appear to be preserved in corresponding xenografts.

In the case of the xenograft 19xa the results of the DNA fingerprinting showed that the xenograft DNA did not match normal DNA (19n) and DNA of another xenograft (19xb) established from the same primary tumour. All precautions were taken to ensure the proper labelling of colorectal cancer xenografts established for the purpose of this study to allow correct identification of the xenografts and their tumours of origin. Additional investigations were undertaken in cases where the possibility of human error could not be excluded. Since analysis of polymorphic microsatellite loci would not be helpful in the case of xenograft 19xa the interpretation relies on the results of the DNA fingerprinting analysis which indicates that sample 19xa has been misidentified. There is no reason to believe that the hypervariable telomeric sequence chosen for the fingerprinting analysis is susceptible to mismatch repair defects. When other RER+ tumours and normal tissue were tested using the same method (3n and 3b, 18n and 18c, see Figure 26a and b), MMR deficiency did not appear to affect the results.

6.5. Summary.

In summary, there seems to be very good correlation between the RER status of primary colorectal cancers and their corresponding xenografts. The RER+ phenotype was preserved in all colorectal cancer xenografts established from RER+ primary tumours. No evidence was found that RER+ phenotype can spontaneously appear in sporadic colorectal cancer during xenograft establishment and passage in SCID mice. Colorectal cancer xenografts represent an excellent study model for involvement of mismatch repair system in response of tumour cells to various therapeutic agents.

CHAPTER 7

Summary and future prospects.

7.1. Summary.

This thesis has analysed genetic intratumoral heterogeneity in sporadic colorectal cancer and its association with two known types of underlying genomic instability. It has also established the relevance of colorectal cancer xenografts as models for investigating the genetics of colorectal cancer. The data gathered will be useful in assessing treatment response in colorectal cancer xenografts to various therapeutic agents in relation to specific genetic abnormalities.

In summary, the most important finding of this study is the identification of a novel group of sporadic colorectal cancers which do not display instability of either chromosomes or microsatellites (called non-MIN, non-CIN cancers). These tumours do not show any striking differences in clinical and pathological features compared with RER- tumours exhibiting high levels of chromosomal instability but may harbour fewer abnormalities of p53. The data also suggest that these cancers display a different pattern of clonal chromosomal abnormalities than high-CIN RER- tumours indicating that a different subset of tumour suppresser genes might be targeted in this tumour group. It is likely that non-MIN, non-CIN colorectal cancers represent a distinct entity in sporadic colorectal cancer. Based on this data their prevalence might be as high as 35% of RER- colorectal cancers, constituting 25% of sporadic colorectal cancers in total. These tumours might harbour one or more novel mechanisms of genomic instability or alternatively they represent a group of sporadic colorectal cancers developing without increased mutation rate thorough selection of advantageous mutation and clonal expansion.

The analysis of chromosome copy number changes in RER- and RER+ groups of sporadic colorectal cancers confirmed previously reported differences in rates of chromosomal abnormalities occurring in these two cancer groups. Different patterns of chromosomal changes were found to occur most frequently in RER- and RER+ tumours. The specific pattern of chromosomal gains and losses identified RER- sporadic colorectal cancers (20q+, 18q-, 13q+, 8p-, 1p- and 8q+) generally conforms

with the results of many previous cytogenetic and CGH studies. However, frequent loss of chromosome 19 in RER+ group of tumours found in this study has not been previously reported. Differences in rates at which chromosomal abnormalities occur together with different patterns of chromosomal changes appearing in RER- and RER+ group of colorectal cancer further support the hypothesis that pathways of carcinogenesis in these two groups of tumours are fundamentally different.

This study has identified the presence of genetic intratumoral heterogeneity in sporadic colorectal cancer. The analysis of colorectal cancer xenografts established from samples collected from multiple sites from primary tumours showed that a xenograft established from a single sample is in general representative of its tumour of origin, despite the presence of genetic heterogeneity within primary tumours. This applies firstly to the preservation of the RER+ and RER- phenotype and secondly to specific chromosomal abnormalities being retained in RER- colorectal cancer xenografts. The study also showed that p53 status of the primary tumour is unchanged in a corresponding xenograft and that the DNA ploidy closely resembles that of the sample of origin. Preservation of all these important genetic features in colorectal cancer xenografts makes them a valuable model for investigating the genetics of the disease.

7.2. Future prospects.

Much remains to be understood about the mechanisms of genomic instability so far discovered in sporadic colorectal cancer. At present little is known about factors determining tumour response to radio- or chemotherapy, although there is increasing evidence connecting treatment response to the specific genetic constitution of the tumour. At biochemical level the mechanisms of action of commonly used anticancer treatments are well understood. X-irradiation, topoisomerase inhibitors and alkylating agents damage DNA. Spindle poisons bind to microtubules and inhibit their function. Antimetabolites disrupt nucleotide pools and thereby inhibit DNA synthesis. However, it remains unclear why these biochemical activities, all of which induce p53 dependent cell-cycle arrest, provide tumour specificity, killing cancer cells more

efficiently than normal cells (Waldman *et al.*, 1997). In experimental systems MMR deficient cells are highly tolerant to the methylating chemotherapeutic drugs streptozocin and temozolamide (Koi *et al.*, 1994; de Wind *et al.*, 1995; Liu *et al.*, 1996a; Umar *et al.*, 1997) and to a lesser extent to cisplatin and doxorubicin (Drummond *et al.*, 1996b; Aebi *et al.*, 1996; Fink *et al.*, 1996). These drugs are therefore expected to work less effectively in treatment of MMR deficient tumours in humans. Understanding of the complex mechanisms involved in the response of tumour cells to various therapeutic agents is crucial. Characterising the genotype-dependent treatment response of colorectal cancers will substantially facilitate the appropriate choice of treatment according to individual needs and will be extremely useful in predicting the outcome of such treatment. This issue has been addressed by other members of this group using the colorectal cancer xenografts characterised in this study. Additionally, it is important to further characterise the novel group of sporadic colorectal cancers which do not display instability of either chromosomes or microsatellites in order to determine whether these tumours harbour other mechanism(s) of genomic instability.

Bibliography

Aaltonen, L.A., Peltomaki, P., Leach, F.S., Sistonen, P., Pylkkanen, L., Mecklin, J.P., Jarvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., Vogelstein, B., and de la Chapelle, A. (1993). Clues to the pathogenesis of familial colorectal cancer. *Science* ***260***, 812-816.

Aaltonen, L.A., Peltomaki, P., Mecklin, J.P., Jarvinen, H., Jass, J.R., Green, J.S., Lynch, H.T., Watson, P., Tallqvist, G., Juhola, M., Sistonen, P., Hamilton, S.R., Kinzler, K.W., Vogelstein, B., and de la Chapelle, A. (1994). Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Research* ***54***, 1645-1648.

Aas, T., Borresen, A.L., Geisler, S., Smith-Sorensen, B., Johnsen, H., Varhaug, J.E., Akslen, L.A., and Lonning, P.E. (1996). Specific p53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nature Medicine* ***2***, 811-814.

Abdel-Rahman, W.M., Georgiades, I.B., Curtis, L.J., Arends, M.J. and Wyllie, A.H. (1999). Role of BAX mutations in mismatch repair-deficient colorectal carcinogenesis. *Oncogene* ***18***, 2139-2142.

Aebi, S., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R.D., Boland, C.R., Koi, M., Fishel, R., and Howell, S.B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Research* ***56***, 3087-3090.

Ahuja, N., Li, Q., Mohan, A.L., Baylin, S.B., and Issa, J.P.J. (1998). Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Research* ***58***, 5489-5494.

Akiyama, Y., Iwanaga, R., Ishikawa, T., Sakamoto, K., Nishi, N., Nihei, Z., Iwama, T., Saitoh, K. and Yuasa, Y. (1996). Mutations of the transforming growth factor-β type II receptor gene are strongly related to sporadic proximal colon carcinomas with microsatellite instability. *Cancer* ***78***, 2478-2484.

Akiyama, Y., Iwanaga, R., Saitoh, K., Shiba, K., Ushio, K., Ikeda, E., Iwama, T., Nomizu, T. and Yuasa, Y. (1997a). Transforming growth factor β type II receptor gene mutations in adenomas from Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology* ***112***, 33-39.

Akiyama, Y., Sato, H., Yamada, T., Nagasaki, H., Tsuchiya, A., Abe, R., and Yuasa, Y. (1997b). Germ-line mutation of the hMSH6/GTBP gene in an atypical hereditary nonpolyposis colorectal cancer kindred. *Cancer Research* ***57***, 3920-3923.

Arzimanoglou, I.I., Gilbert, F., and Barber, H.R.K. (1998). Microsatellite instability in human solid tumors. *Cancer* ***82***, 1808-1820.

Auer, G.U., Heselmeyer, K.M., Steinbeck, R.G., Munckwikland, E., and Zetterberg, A.D. (1994). The relationship between aneuploidy and p53 overexpression during genesis of colorectal adenocarcinoma. *Virchows Archiv-An International Journal of Pathology* ***424***, 343-347.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K.V., Hamilton, S., and Vogelstein, B. (1990). P53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Research* ***50***, 7717-7722.

Bardi, G., Johansson, B., Pandis, N., Bakjensen, E., Orndal, C., Heim, S., Mandahl, N., Andrensandberg, A., and Mitelman, F. (1993a). Cytogenetic aberrations in colorectal adenocarcinomas and their correlation with clinicopathological features. *Cancer* ***71***, 306-314.

Bardi, G., Johansson, B., Pandis, N., Mandahl, N., Bakjensen, E., Lindstrom, C., Tornqvist, A., Frederiksen, H., Andrensandberg, A., Mitelman, F., and Heim, S. (1993b). Cytogenetic analysis of 52 colorectal carcinomas - nonrandom aberration pattern and correlation with pathological parameters. *International Journal of Cancer* ***55***, 422-428.

Bardi, G., Parada, L.A., Bomme, L., Pandis, N., Johansson, B., Willen, R., Fenger, C., Kronborg, O., Mitelman, F., and Heim, S. (1997a). Cytogenetic findings in metastases from colorectal cancer. *International Journal of Cancer* ***72***, 604-607.

Bardi, G., Parada, L.A., Bomme, L., Pandis, N., Willen, R., Johansson, B., Jeppsson, B., Beroukas, K., Heim, S., and Mitelman, F. (1997b). Cytogenetic comparisons of synchronous carcinomas and polyps in patients with colorectal cancer. *British Journal of Cancer* ***76***, 765-769.

Bardi, G., Sukhikh, T., Pandis, N., Fenger, C., Kronborg, O., and Heim, S. (1995). Karyotypic characterization of colorectal adenocarcinomas. *Genes Chromosomes & Cancer* ***12***, 97-109.

Barletta, C., Scillato, F., Sega, F.M., and Mannella, E. (1993). Genetic alteration in gastrointestinal cancer - a molecular and cytogenetic study. *Anticancer Research* ***13***, 2325-2329.

Baylin, S.B., Herman, J.G., Graff, J.R., Vertino, P.M. and Issa, J.P. (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia. *Advances in Cancer Research* ***72***, 141-196.

Bebenek, K. and Kunkel, T.A. (1990). Frameshift errors initiated by nucleotide misincorporation. *Proceedings of the National Academy of Sciences of the United States of America* ***87***, 4946-4950.

Belinsky, S.A., Nikula, K.J., Palmisano, W.A., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S.B., and Herman, J.G. (1998). Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proceedings of the National Academy of Sciences of the United States of America* ***95***, 11891-11896.

Bell, S.M., Scott, N., Cross, D., Sagar, P., Lewis, F.A., Blair, G.E., Taylor, G.R., Dixon, M.F., and Quirke, P. (1993). Prognostic value of p53 overexpression and c-ki-ras gene-mutations in colorectal cancer. *Gastroenterology* ***104***, 57-64.

Bhattacharyya, N.P., Ganesh, A., Phear, G., Richards, B., Skandalis, A. and Meuth, M. (1995). Molecular analysis of mutations in mutator colorectal carcinoma cell lines. *Human Molecular Genetics* ***4***, 2057-2064.

Bhattacharyya, N.P., Skandalis, A., Ganesh, A., Groden, J. and Meuth, M. (1994). Mutator phenotypes in human colorectal carcinoma cell lines. *Proceedings of the National Academy of Sciences of the United States of America* ***91***, 6319-6323.

Bischoff, F.Z., Yim, S.O., Pathak, S., Grant, G., Siciliano, M.J., Giovanella, B.C., Strong, L.C., and Tainsky, M.A. (1990). Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome - aneuploidy and immortalization. *Cancer Research* ***50***, 7979-7984.

Bischoff, J.R., Anderson, L., Zhu, Y.F., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C.S.M., Novotny, M., Slamon, D.J., and Plowman, G.D. (1998). A homologue of drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *Embo Journal* ***17***, 3052-3065.

Bodmer, W.F., Bailey, C.J., Bodmer, J., Bussey, H.J., Ellis, A., Gorman, P., Lucibello, F.C., Murday, V.A., Rider, S.H. and Scambler, P. (1987). Localization of the gene for familial adenomatous polyposis on chrmosome 5. *Nature* ***328***, 614-616.

Boland, C.R., Sato, J., Saito, K., Carethers, J.M., Marra, G., Laghi, L., and Chauhan, D.P. (1998). Genetic instability and chromosomal aberrations in colorectal cancer: a review of the current models. *Cancer Detection and Prevention* ***22***, 377-382.

Bomme, L., Bardi, G., Pandis, N., Fenger, C., Kronborg, O., and Heim, S. (1996). Chromosome abnormalities in colorectal adenomas: two cytogenetic subgroups characterized by deletion of 1p and numerical aberrations. *Human Pathology* ***27***, 1192-1197.

Bomme, L., Bardi, G., Pandis, N., Fenger, C., Kronborg, O., and Heim, S. (1998). Cytogenetic analysis of colorectal adenomas: karyotypic comparisons of synchronous tumors. *Cancer Genetics and Cytogenetics* ***106***, 66-71.

Borresen, A.L., Lothe, R.A., Meling, G.I., Lystad, S., Morrison, P., Lipford, J., Kane, M.F., Rognum, T.O., and Kolodner, R.D. (1995). Somatic mutations in the hMSH2 gene in microsatellite unstable colorectal carcinomas. *Human Molecular Genetics* ***4***, 2065-2072.

Bosari, S., Viale, G., Roncalli, M., Graziani, D., Borsani, G., Lee, A.K.C., and Coggi, G. (1995). P53 gene mutations, p53 protein accumulation and compartmentalization in colorectal adenocarcinoma. *American Journal of Pathology* ***147***, 790-798.

Bosma, M., Schuler, W. and Bosma, G. (1988). The scid mouse mutant. *Current Topics in Microbiology and Immunology* ***137***, 197-202.

Bouffler, S.D., Kemp, C.J., Balmain, A., and Cox, R. (1995). Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. *Cancer Research* ***55***, 3883-3889.

Boyer, J.C., Umar, A., Risinger, J.I., Lipford, J.R., Kane, M., Yin, S., Barrett, J.C., Kolodner, R.D. and Kunkel, T.A. (1995). Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. *Cancer Research* ***55***, 6063-6070.

Bradley, B.A. and Evers, B.M. (1997). Molecular advances in the etiology and treatment of colorectal cancer. *Surgical Oncology-Oxford* ***6***, 143-156.

Branch, P., Aquilina, G., Bignami, M., and Karran, P. (1993). Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature* ***362***, 652-654.

Branch, P., Hampson, R., and Karran, P. (1995). DNA mismatch binding defects, DNA damage tolerance, and mutator phenotypes in human colorectal carcinoma cell lines. *Cancer Research* ***55***, 2304-2309.

Breivik, J. and Gaudernack, G. (1999). Carcinogenesis and natural selection: a new perspective to the genetics and epigenetics of colorectal cancer. *Advances in Cancer Research* ***76***, 187-212.

Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag, R.J., Godwin, A.R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. (1994). Mutation in the DNA mismatch repair gene homolog hMSH1 is associated with hereditary nonpolyposis colon cancer. *Nature* ***368***, 258-261.

Bumpers, H.L., Alosco, T.R., Wang, H.Q., Petrelli, N.J., Hoover, E.L., and Bankert, R.B. (1994). Human villous adenomas engrafted into acid mice survive for prolonged period without malignant transformation. *Journal of Clinical Investigation* ***94***, 2153-2157.

Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K.V., Markowitz, S.D., Kinzler, K.W., and Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature* ***392***, 300-303.

Cancer Research Campaign Scientific Yearbook, 1998-99.

Carder, P., Wyllie, A.H., Purdie, C.A., Morris, R.G., White, S., Piris, J., and Bird, C.C. (1993). Stabilized p53 facilitates aneuploid clonal divergence in colorectal cancer. *Oncogene* ***8***, 1397-1401.

Carder, P.J., Cripps, K.J., Morris, R., Collins, S., White, S., Bird, C.C., and Wyllie, A.H. (1995). Mutation of the p53 gene precedes aneuploid clonal divergence in colorectal carcinoma. *British Journal of Cancer* ***71***, 215-218.

Carder, P.J., Purdie, C.A., Piris, J., Wyllie, A.H., and Bird, C.C. (1991). P53 and malignant transformation in colorectal neoplasia. *Journal of Pathology* ***164***, A364

Cawkwell, L., Bell, S.M., Lewis, F.A., Dixon, M.F., Taylor, G.R., and Quirke, P. (1993). Rapid detection of allele loss in colorectal tumors using microsatellites and fluorescent DNA technology. *British Journal of Cancer* ***67***, 1262-1267.

Chen, R.Z., Pettersson, U., Beard, C., Jackson-Grusby, L., and Jaenisch, R. (1998). DNA hypomethylation leads to elevated mutation rates. *Nature* ***395***, 89-93.

Cho, K.R., Oliner, J.D., Simons, J.W., Hedrick, L., Fearon, E.R., Preisinger, A.C., Hedge, P., Silverman, G.A., and Vogelstein, B. (1994). The DCC gene - structural analysis and mutations in colorectal carcinomas. *Genomics* ***19***, 525-531.

Chong, J.M., Fukayama, M., Hayashi, Y., Takizawa, T., Koike, M., Konishi, M., Kikuchi-Yanoshita, R., and Miyaki, M. (1994). Microsatellite instability in the progression of gastric carcinoma. *Cancer Research* ***54***, 4595-4597.

Chuong, C.M., Jiang, T.X., Tin, E. and Widelitz, R.B. (1994). cDCC (chicken homologue to a gene deleted in colorectal carcinima) is an epithelial adhesion molecule expressed in the basal cells and involved in epithelial-mesenchymal interaction. *Developmental Biology* ***164***, 383-97.

Clarke, A.R., Howard, L.A., Harrison, D.J., and Winton, D.J. (1997). P53 and mutation rate: a relationship in the murine intestine only at high levels of DNA damage. *Journal of Pathology* ***181***, A33

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* ***362***, 849-852.

Cleary, M.L. and Smith, S.D. (1986). Cloning and structural analysis of cDNAs for bcl-2 and hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* ***47***, 19-28.

Cooke, H.J., Brown, W.R.A., and Rappold, G.A. (1985). Hypervariable telomeric sequences from the human sex-chromosomes are pseudoautosomal. *Nature* ***317***, 687-692.

Corbet, S.W., Clarke, A.R., Gledhill, S. and Wyllie, A.H. (1999). P53-dependent and -independent DNA-damage, apoptosis and mutation frequency in ES cells. *Oncogene* ***18***,

Costa, A., Marasca, R., Valentinis, B., Savarino, M., Faranda, A., Silvestrini, R., and Torelli, G. (1995). P53 gene point mutations in relation to p53 nuclear protein accumulation in colorectal cancers. *Journal of Pathology* ***176***, 45-53.

Cottu, P.H., Muzeau, F., Estreicher, A., Flejou, J.F., Iggo, R., Thomas, G., and Hamelin, R. (1996). Inverse correlation between RER(+) status and p53 mutation in colorectal cancer cell lines. *Oncogene* ***13***, 2727-2730.

Cowin, P. (1994). Unraveling the cytoplasmic interactions of catherin superfamily. *Proceedings of the National Academy of Sciences of the United States of America* ***91***, 10759-10761.

Counts, J.L. and Goodman, J.I. (1995). Hypomethylation of DNA: a possible epigenetic mechanism involved in tumor promotion. *Progress in Clinical and Biological Research* ***391***, 81-101.

Couturier-Turpin, M.H., Esnous, C., Louvel, A., Poirier, Y. Couturier, D. (1992). Chromosome 1 in human colorectal tumors. Cytogenetic research on structural changes and their significance. *Human Genetics* ***88***, 431-438.

Crawford, J.M. The Gastrointerstinal Tract. In Cotran, R.S, Kumar, V. and Collins, T. eds. *Robbins Pathologic Basis of Disease*. Philadelphia: W.B. Saunders Company, 1999, 775-844.

Cripps, K.J., Purdie, C.A., Carder, P.J., White, S., Komine, K., Bird, C.C., and Wyllie, A.H. (1994). A study of stabilization of p53 protein versus point mutation in colorectal carcinoma. *Oncogene* ***9***, 2739-2743.

Cripps, K.J., White, S., Komine, K., Carder, P.J., Purdie, C.A., Wyllie, A.H., and Bird, C.C. (1994). A comparative study of PCR-SSCP, sequencing and immunocytochemistry, in the detection of p53 mutations in colorectal cancer. *Journal of Cellular Biochemistry* ***199***,

Crowcroft, N.S., Vyse, A., Brown, D.W.G., and Strachan, D.P. (1998). Epidemiology of Epstein-Barr virus infection in pre-adolescent children: application of a new salivary method in Edinburgh, Scotland. *Journal of Epidemiology and Community Health* ***52***, 101-104.

Cunningham, C., Dunlop, M.G., Wyllie, A.H., and Bird, C.C. (1993). Deletion mapping in colorectal cancer of a putative tumor suppressor gene in 8p22-p21.3. *Oncogene* ***8***, 1391-1396.

Cunningham, J., Lust, J.A., Schaid, D.J., Bren, G.D., Carpenter, H.A., Rizza, E., Kovach, J.S., and Thibodeau, S.N. (1992). Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Research* ***52***, 1974-1980.

Cunningham, J.M., Christensen, E.R., Tester, D.J., Kim, C.Y., Roche, P.C., Burgart, L.J. and Thibodeau, S.N. (1998). Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Research* ***58***, 3455-3460.

Curtis, L.J.(1998).Genomic instability in sporadic colorectal cancer. PhD thesis. University of Edinburgh.

Curtis, L.J., Bubb, V.J., Gledhill, S., Morris, R.G., Bird, C.C. and Wyllie, A.H. (1994). Loss of heterozygosity of MMC is not associated with mutation of the retained allele in sporadic colorectal cancer. *Human Molecular Genetics* ***3***, 443-446.

Deangelis, P., Stokke, T., Smedshammer, L., Lothe, R.A., Meling, G.I., Rofstad, M., Chen, Y., and Clausen, O.P.F. (1993). P53 expression is associated with a high degree of tumor DNA aneuploidy and incidence of p53 gene mutation, and is localized to the aneuploid component in colorectal carcinomas. *International Journal of Oncology* ***3***, 305-312.

Deng, G., Chen, A., Hong, J., Chae, H.S. and Kim, Y.S. (1999). Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the abscence of gene expression. *Cancer Research* ***59***, 2029-2033.

de Wind, N., Dekker, M., Berns, A., Radman, M., and Riele, H.T. (1995). Inactivation of the mouse MSH2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* ***82***, 321-330.

di Vinci, A., Infusini, E., Peveri, C., Risio, M., Rossini, F.P., and Giaretti, W. (1996). Deletions at chromosome 1p by fluorescence in situ hybridization are an early event in human colorectal tumorigenesis. *Gastroenterology* ***111***, 102-107.

Dix, B., Robbins, P., Carrello, S., House, A., and Iacopetta, B. (1994). Comparison of p53 gene mutation and protein overexpression in colorectal carcinomas. *British Journal of Cancer* ***70***, 585-590.

Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.P., Pinkel, D., Gray, T., Bradley, A., Medina, D., and Varmus, H.E. (1995). Deficiency of p53 accelerates mammary tumorigenesis in WNT-1 transgenic mice and promotes chromosomal instability. *Genes & Development* ***9***, 882-895.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A.Jr., Butel, J.S. and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* ***356***, 215-221.

Dong, S.M., Kim, K.M., Kim, S.Y., Shin, M.S., Na, E.Y., Lee, S.H., Park, W.S., Yoo, N.J., Jang, J.J., Yoon, C.Y., Kim, J.W., Yang, Y.M., Kim, S.H., Kim, C.S., and Lee, J.Y. (1998). Frequent somatic mutations in serine/threonine kinase 11 Peutz-Jeghers syndrome gene in left-sided colon cancer. *Cancer Research* ***58***, 3787-3790.

Doxsey, S. (1998). The centrosome - a tiny organelle with big potential. *Nature Genetics* ***20***, 104-106.

Drummond, J., Li, G.M., Duckett, D., and Modrich, P. (1996a). HmutL alpha and hmutS alpha in human mismatch repair. *Faseb Journal* ***10***, 9.

Drummond, J.T., Anthoney, A., Brown, R., and Modrich, P. (1996b). Cisplatin and adriamycin resistance are associated with mutL alpha and mismatch repair deficiency in an ovarian tumor cell line. *Journal of Biological Chemistry* ***271***, 19645-19648.

Duckett, D.R., Drummond, J.T., Murchie, A.I.H., Reardon, J.T., Sancar, A., Lilley, D.M., and Modrich, P. (1996). Human mutS alpha recognizes damaged DNA base pairs containing o-6-methylguanine, o-4-methylthymine, or the cisplatin-d(gpg) adduct. *Proceedings of the National Academy of Sciences of the United States of America* ***93***, 6443-6447.

Dunlop, M.G., Farrington, S.M., Bubb, V.J., Cunningham, C., Wright, M., Curtis, L.J., Butt, Z.A., Wright, E., Fleck, B.W., Redhead, D., Mitchell, R., Rainey, J.B., Macintyre, I.M.C., Carter, D.C., and Wyllie, A.H. (1996). Extracolonic features of familial adenomatous polyposis in patients with sporadic colorectal cancer. *British Journal of Cancer* ***74***, 1789-1795.

el-Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E. and Wang, Y. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Research* ***54***, 1169-1174.

Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik,Y., Arai, N., Rotter, V. and Oren, M. (1988). Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene* ***3***, 131-321.

Elledge, S.J. (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* ***274***, 1664-1672.

Eppert, K., Scherer, S.W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L.C., Bapat, B., Gallinger, S., Andrulis, I.L., Thomsen, G.H., Wrana, J.L., and Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGF beta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* ***86***, 543-552.

Eshleman, J.R., Casey, G., Kochera, M.E., Sedwick, W.D., Swinler, S.E., Veigl, M.L., Willson, J.K.V., Schwartz, S., and Markowitz, S.D. (1998a). Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of p53. *Oncogene* ***17***, 719-725.

Eshleman, J.R., Donover, P.S., Littman, S.J., Swinler, S.E., Li, G.M., Lutterbaugh, J.D., Willson, J.K.V., Modrich, P., Sedwick, W.D., Markowitz, S.D., and Veigl, M.L. (1998b). Increased transversions in a novel mutator colon cancer cell line. *Oncogene* ***16***, 1125-1130.

Eshleman, J.R., Lang, E.Z., Bowerfind, G.K., Parsons, R., Vogelstein, B., Willson, J.K.V., Veigl, M.L., Sedwick, W.D., and Markowitz, S.D. (1995). Increased mutation rate at the *hprt* locus accompanies microsatellite instability in colon cancer. *Oncogene* ***10***, 33-37.

Eshleman, J.R. and Markowitz, S.D. (1996). Mismatch repair defects in human carcinogenesis. *Human Molecular Genetics* ***5***, 1489-1494.

Eshleman, J.R., Markowitz, S.D., Donover, P.S., Lang, E.Z., Lutterbaugh, J.D., Li, G.M., Longley, M., Modrich, P., Veigl, M.L., and Sedwick, W.D. (1996). Diverse hypermutability of multiple expressed sequence motifs present in a cancer with microsatellite instability. *Oncogene* ***12***, 1425-1432.

Farrington, S.M., Cunningham, C., Boyle, S.M., Wyllie, A.H., and Dunlop, M.G. (1996). Detailed physical and deletion mapping of 8p with isolation of YAC clones from tumour suppressor loci involved in colorectal cancer. *Oncogene* ***12***, 1803-1808.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M., and Weinberg, R.A. (1997). Phenotype of mice lacking functional deleted in colorectal cancer (DCC) gene. *Nature* ***386***, 796-804.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W., and Vogelstein, B. (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* ***247***, 49-56.

Fearon, E.R. and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* ***61***, 759-767.

Feil, R. and Kelsey, G. (1997). Genomic imprinting: a chromatin connection. *American Journal of Human Genetics* ***61***, 1213-1219.

Feinberg, A.P. (1988). Alterations in DNA methylation in colorectal polyps and cancer. *Progress in Clinical and Biological Research* ***279***, 309-317.

Feunteun, J. (1998). Breast cancer and genetic instability: the molecules behind the scene. *Molecular Medicine Today* ***4***, 263-267.

Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R.D. and Howell, S.B. (1996). The role of DNA mismatch repair in platinum drug resistance. *Cancer Research* ***56***, 4881-4886.

Fritsche, M., Haessler, C. and Brander, G. (1993). Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* ***8***, 307-318.

Fujiwara, Y., Emi, M., Ohata, H., Kato, Y., Nakajima, T., Mori, T. and Nakamura, Y. (1993). Evidence for the presence of 2 tumor suppressor genes on chromosome 8p for colorectal carcinoma. *Cancer Research* ***53***, 1172-1174.

Fuzzati-Armentero, M.T. and Duchosal, M.A. (1998). Hu-PBL-SCID mice: an in vivo model of Epstein-Barr virus-dependent lymphoproliferative disease. *Histology and Histopathology* ***13***, 155-168.

Garnier, J.L., Cannon, M.J., Chargui, J., Touraine, J.L., and Cooper, N.R. (1993). EBV human B-cell lymphomas in SCID mice - description of the model and study of the role of anti-CD40 and anti-CD23 monoclonal antibodies in their development. *Transplantation Proceedings* ***25***, 1387-1388.

Gerdes, H., Chen, Q.G., Elahi, A.H., Sircar, A., Goldberg, E., Winawer, D., Urmacher, C., Winawer, S.J., and Jhanwar, S.C. (1995). Recurrent deletions involving chromosome 1, chromosome 5, chromosome 17, and chromosome 18 in colorectal carcinoma - possible role in biological and clinical behavior of tumors. *Anticancer Research* ***15***, 13-24.

Giaretti, W., Monaco, R., Pujic, N., Rapallo, A., Nigro, S., and Geido, E. (1996). Intratumor heterogeneity of K-ras2 mutations in colorectal adenocarcinomas. *American Journal of Pathology* ***149***, 237-245.

Giovannucci, E. and Willett, W.C. (1994). Dietary factors and risk of colon cancer. *Annals of Medicine* ***26***, 443-452.

Goelz, S.E., Hamilton, S.R., and Vogelstein, B. (1985a). Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochemical and Biophysical Research Communications* ***130***, 118-126.

Goelz, S.A., Vogelstein, B., Hamilton, S.R. and Feinberg, A.P. (1985b). Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* ***228***, 187-190.

Goh, H.S., Chan, C.S., Khine, K., and Smith, D.R. (1994). P53 and behavior of colorectal cancer. *Lancet* ***344***, 233-234.

Gottlieb, T.M. and Oren, M. (1996). P53 in growth control and neoplasia. *Biochimica Et Biophysica Acta Reviews on Cancer* ***1287***, 77-102.

Grady, W.M., Myeroff, L.L., Swinler, S.E., Rajput, A., Thiagalingam, S., Lutterbaugh, J.D., Neumann, A., Brattain, M.G., Chang, J., Kim, S-J., Kinzler, K.W., Vogelstein, B., Willson, J.K.V. and Markowitz, S. (1999). Mutational inactivation of transforming growth factor β receptor type II in microsatellte stable colon cancers. *Cancer Research* ***59***, 320-324.

Griffin, C.A., Lazar, S., Hamilton, S.R., Giardiello, F.M., Long, P., Krush, A.J., and Booker, S.V. (1993). Cytogenetic analysis of intestinal polyps in polyposis syndromes - comparison with sporadic colorectal adenomas. *Cancer Genetics and Cytogenetics* ***67***, 14-20.

Griffiths, S.D., Clarke, A.R., Healy, L.E., Ross, G., Ford, A.M., Hooper, M.L., Wyllie, A.H., and Greaves, M. (1997). Absence of p53 permits propagation of mutant cells following genotoxic damage. *Oncogene* ***14***, 523-531.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spiro, L. and Robertson, M. (1991). Identification and characterization of the familial polyposis coli gene. *Cell* ***66***, 589-600.

Gualberto, A., Aldape, K., and Kozakiewicz, K. (1998). An oncogenic form of p53 confers a dominant, gain of function phenotype that disrupts spindle checkpoint control. *Proceedings of the National Academy of Sciences of the United States of America* ***95***, 5166-5171.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. (1994). The 1993-94 genethon human genetic linkage map. *Nature Genetics 7*, 246-339.

Habano, W., Nakamura, S., and Sugai, T. (1998). Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome. *Oncogene* ***17***, 1931-1937.

Hamelin, R., Laurentpuig, P., Olschwang, S., Jego, N., Asselain, B., Remvikos, Y., Girodet, J., Salmon, R.J., and Thomas, G. (1994). Association of p53 mutations with short survival in colorectal cancer. *Gastroenterology* ***106***, 42-48.

Han, S., Semba, S., Abe, T., Makino, N., Furukawa, T., Fukushige, S., Takahashi, H., Sakurada, A., Sato, M., Shiiba, K., Matsuno, S., Nimura, Y., Nakagawara, A. and Horii, A. (1999). Infrequent somatic mutations of the p73 gene in various human cancers. *European Journal of Clinical Oncology* ***25***, 194-198.

Haque, T., Iliadou, P., Hossain, A., and Crawford, D.H. (1996). Seroepidemiological study of Epstein-Barr virus infection in Bangladesh. *Journal of Medical Virology* ***48***, 17-21.

Hartwell, L. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* ***71***, 543-546.

Harvey, M., Sands, A.T., Weiss, R.S., Hegi, M.E., Wiseman, R.W., Pantazis, P., Giovanella, B.C., Tainsky, M.A., Bradley, A., and Donehower, L.A. (1993). In-vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* ***8***, 2457-2467.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B and Kinzler, K.W. (1998). Identification of c-myc as a target of the APC pathway. *Science* ***281***, 1509-1512.

Hedrick, L., Cho, K.R., Fearon, E.R., Wu, T.C., Kinzler, K.W., and Vogelstein, B. (1994). The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes & Development* ***8***, 1174-1183.

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Hoglund, P., Jarvinen, H., Kristo, P., Pelin, K., Ridanpaa, M., Salovaara, R., Toro, T., Bodmer, W., Olschwang, S., Olsen, A.S., Stratton, M.R., de la Chapelle, A., and Aaltonen, L.A. (1998). A serine/threonine kinase gene defective in Peutz-Jegheus syndrome. *Nature* ***391***, 184-187.

Hemminki, A., Tomlinson, I., Markie, D., Jarvinen, H., Sistonen, P., Bjorkqvist, A.M., Knuutila, S., Salovaara, R., Bodmer, W., Shibata, D., de la Chapelle, A., and Aaltonen, L.A. (1997). Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. *Nature Genetics* ***15***, 87-90.

Herberg, F.W., Bell, S.M., and Taylor, S.S. (1993). Expression of the catalytic subunit of cAMP-dependent protein kinase in Escherichia Coli - multiple isozymes reflect different phosphorylation states. *Protein Engineering* ***6***, 771-777.

Herbergs, J., Debruine, A.P., Marx, P.T.J., Vallinga, M.I.J., Stockbrugger, R.W., Ramaekers, F.C.S., Arends, J.W., and Hopman, A.H.N. (1994). Chromosome aberrations in adenomas of the colon - proof of trisomy 7 in tumor cells by combined interphase cytogenetics and immunocytochemistry. *International Journal of Cancer* ***57***, 781-785.

Herbergs, J., Hopman, A.H.N., Debruine, A.P., Ramaekers, F.C.S., and Arends, J.W. (1996). In situ hybridization and flow cytometric analysis of colorectal tumours suggests two routes of tumourigenesis characterized by gain of chromosome 7 or loss of chromosomes 17 and 18. *Journal of Pathology* ***179***, 243-247.

Herman, J.G., Umar, A., polyak, K., Graff, J.R., Ahuja, N., Issa, J.P., Markowitz, S., Willson, J.K., Hamilton, S.R., Kinzler, K.W., Kane, M.F., Kolodner, R.O., Vogelstein, B., Kunkel, T.A. and Baylin, S.B. (1998). Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* ***95***, 6870-6875.

Herrera, L., Kakati, S., Gibas, L., Pietrzak, E. and Sadnberg, A.A. (1986). Gardner syndrome in a man with an interstitial deletion of 5q. *American Journal of Medical Genetics* ***25***, 473-476.

Hoang, J.M., Cottu, P.H., Thuille, B., Salmon, R.J., Thomas, G., and Hamelin, R. (1997). BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Research* ***57***, 300-303.

Hoffmann, J.S. and Cazaux, C. (1998). DNA synthesis, mismatch repair and cancer. *International Journal of Oncology* ***12***, 377-382.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). P53 mutations in human cancers. *Science* ***253***, 49-53.

Hoosein, N.M., McKnight, M.K., Levine, A.E., Mulder, K.M., Childress, K.E., Brattain, D.E. and Brattain, M.G. (1989). Differential sensitivity of subclass of human colon carcinoma cell lines to the growth inhibitory effects of transforming growth factor-β_1. *Experimental Cell Research* ***181***, 442-453.

Howe, J.R. and Guillem, J.G. (1997). The genetics of colorectal cancer. *Surgical Clinics of North America 77*, 175-195.

Huang, J., Papadopoulos, N., McKinley, A.J., Farrington, S.M., Curtis, L.J., Wyllie, A.H., Zheng, S., Willson, J.K.V., Markowitz, S.D., Morin, P., Kinzler, K.W., Vogelstein, B., and Dunlop, M.G. (1996). APC mutations in colorectal tumors with mismatch repair deficiency. *Proceedings of the National Academy of Sciences of the United States of America* ***93***, 9049-9054.

Ilyas, M., Tomlinson, I.P.M., Novelli, M.R., Hanby, A., Bodmer, W.F., and Talbot, I.C. (1996). Clinico-pathological features and p53 expression in left-sided sporadic colorectal cancers with and without microsatellite instability. *Journal of Pathology* ***179***, 370-375.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* ***363***, 558-561.

Ishii, H., Baffa, R., Numata, S.I., Murakumo, Y., Rattan, S., Inoue, H., Mori, M., Fidanza, V., Alder, H. and Croce, C.M. (1999). The FEZ1 gene at chromosome 8p22 encodes a leucine-zipper protein, and its expression is altered in multiple human tumours. *Proceedings of the National Academy of Sciences of the United States of America* ***96***, 3928-3933.

Isobe, M., Emanuel, B.S., Givol, D., Oren, M. and Croce, C.M. (1986). Localization of gene p53 tumour antigen to band 17p13. *Nature* ***320***, 84-85.

Itoh, F., Hinoda, Y., Ohe, M., Ohe, Y., Ban, T., Endo, T., Imai, K., and Yachi, A. (1993). Decreased expression of DCC messenger-RNA in human colorectal cancers. *International Journal of Cancer* ***53***, 260-263.

Iwabuchi, H., Sakamoto, M., Sakunaga, H., Ma, Y.Y., Carcangiu, M.L., Pinkel, D., Yangfeng, T.L., and Gray, J.W. (1995). Genetic analysis of benign, low-grade, and high-grade ovarian tumors. *Cancer Research* ***55***, 6172-6180.

Jackson, A.L. and Loeb, L.A. (1998). The mutation rate and cancer. *Genetics* ***148***, 1483-1490.

Jass, J.R. (1998). Diagnosis of hereditary non-polyposis colorectal cancer. *Histopathology* ***32***, 491-497.

Jass, J.R., Do, K.A., Simms, L.A., Iino, H., Wynter, C., Pillay, S.P., Searle, J., Radford-Smith, G., Young, J., and Leggett, B. (1998). Morphology of sporadic colorectal cancer with DNA replication errors. *Gut* ***42***, 673-679.

Jen, J., Kim, H., Piantadosi, S., Liu, Z.F., Levitt, R.C., Sistonen, P., Kinzler, K.W., Vogelstein, B. and Hamilton, S.R. (1994). Allelic loss of chromosome 18q and prognosis in colorectal cancer. *New England Journal of Medicine* ***33***, 213-221.

Jenne, D.E., Reimann, H., Nezu, J., Friedel, W., Loff, S., Jeschke, R., Muller, D., Back, W., and Zimmer, M. (1998). Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. *Nature Genetics* ***18***, 38-44.

Jeyaprakash, A., Das-Gupta, R., and Kolodner, R. (1996). Saccharomyces cerevisiae PMS2 mutations are alleles of MLH1, and PMS2-2 corresponds to a hereditary nonpolyposis colorectal carcinoma causing missense mutation. *Molecular and Cellular Biology* ***16***, 3008-3011.

Jones, P.A. and Gonzalgo, M.L. (1997). Altered DNA methylation and genome instability: a new pathway to cancer? *Proceedings of the National Academy of Sciences of the United States of America* ***94***, 2103-2105.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F. and Caput, D. (1997). Monoalleliclly expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* ***90***, 809-819.

Kallioniemi, A., Kallioniemi, O.P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H.S., Pinkel, D., Gray, J.W., and Waldman, F.M. (1994). Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proceedings of the National Academy of Sciences of the United States of America* ***91***, 2156-2160.

Kallioniemi, A., Kallioniemi, O.P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F., and Pinkel, D. (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* ***258***, 818-821.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Research* ***51***, 6304-6311.

Kastan, M.B., Zhan, Q.M., Eldeiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., and Fornace, A.J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* ***71***, 587-597.

Kat, A., Thilly, W.G., Fang, W.H., Longley, M.J., Li, G.M., and Modrich, P. (1993). An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. *Proceedings of the National Academy of Sciences of the United States of America* ***90***, 6424-6428.

Kelemen, P.R., Yaremko, M.L., Kim, A., Minna, J., and Westbrook, C.A. (1994a). Chromosome 8p loss of heterozygosity defined by microsatellite repeat markers - frequent deletions in colorectal and lung carcinomas. *Journal of Cellular Biochemistry 195.*

Kelemen, P.R., Yaremko, M.L., Kim, A.H., Montag, A., Michelassi, F., and Westbrook, C.A. (1994b). Loss of heterozygosity in 8p is associated with microinvasion in colorectal carcinoma. *Genes Chromosomes & Cancer* ***11***, 195-198.

Kern, S., Bos, J.L., Fearon, E.R., Vogelstein, B., and Hamilton, S.R. (1988). The relationship between *ras* gene mutations and the adenoma-carcinoma sequence in the colorectum. *Modern Pathology* ***1***, A47

Kern, S.E., Fearon, E.R., Tersmette, K.W., Enterline, J.P., Leppert, M., Nakamura, Y., White, R., Vogelstein, B. and Hamilton, S.R. (1989). Clinical and pathological associations with allelic loss in colorectal carcinoma. *Journal of the American Medical Association* ***261***, 3099-3103.

Khine, K., Smith,D.R. and Goh, H.S. (1994). High frequency of allelic deletion on chromosome 17p in advanced colorectal cancer. *Cancer* ***73***, 28-35.

Kim, H.G., Jen, J., Vogelstein, B., and Hamilton, S.R. (1994). Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *American Journal of Pathology* ***145***, 148-156.

Kinzler, K.W., Nilbert, M.C., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hamilton, S.R., Hedge, P and Markham, A. (1991). Identification of a gene located at 5q21 that is mutated in colorectal cancers. *Science* ***251***, 1366-1370.

Kinzler, K.W. and Vogelstein, B. (1997). Cancer susceptibility genes - gatekeepers and caretakers. *Nature* ***386***, 761-762.

Knudson, A.G. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America* ***68***, 820-823.

Knuutila, S., Aalto, Y., Autio, K., Bjorkqvist, A.M., El-Rifai, W., Hemmer, S., Huhta, T., Kettunen, E., Kiuru-Kuhlefelt, S., Larramendy, M.L., Lushnikova, T., Monni, O., Pere, H., Tapper, J., Tarkkanen, M., Varis, A., Wasenius, V.M., Wolf, M. and Zhu, Y. (1999). DNA copy number losses in human neoplasms. *American Journal of Pathology* ***155***, 683-694.

Koi, M., Umar, A., Chahan, D.P., Cherian, S.P., Carethers, J.M., Kinkel, T.A. and Boland, C.R. (1994). Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine tolerance in colon tumour cells with homozygous hMLH1 mutation. *Cancer Research* ***54***, 4308-4312.

Kolodner, R.D. (1995). Mismatch repair: mechanisms and relationship to cancer susceptibility. *Trends in Biochemical Sciences* ***20***, 397-401.

Kolodner, R. (1996). Biochemistry and genetics of eukaryotic mismatch repair. *Genes & Development* ***10***, 1433-1442.

Konishi, M., Kikuchi-Yanoshita, R., Tanaka, K., Muraoka, M., Onda, A., Okumura, Y., Kishi, N., Iwama, T., Mori, T., Koike, M., Ushio, K., Chiba, M., Nomizu, S., Konishi, F., Utsunomiya, J., and Miyaki, M. (1996). Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology* ***111***, 307-317.

Konstantinova, L.N., Fleischman, E.W., Knisch, V.I., Perevozchikov, A.G., and Kopnin, B.P. (1991). Karyotype pecularities of human colorectal adenocarcinomas. *Human Genetics* ***86***, 491-496.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., and Kastan, M.B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proceedings of the National Academy of Sciences of the United States of America* ***89***, 7491-7495.

Kunkel, T.A. (1990). Misalignment mediated DNA synthesis errors. *Biochemistry* ***29***, 8003-8011.

Kunkel, T.A. (1995). DNA mismatch repair - the intricacies of eukaryotic spell checking. *Current Biology* ***5***, 1091-1094.

Laird, P.W. (1997). Oncogenic mechanisms mediated by DNA methylation. *Molecular Medicine Today* ***3***, 223-229.

Laird, P.W. and Jaenisch, R. (1996). The role of DNA methylation in cancer genetics and epigenetics. *Annual Review of Genetics* ***30***, 441-464.

Lane, D. (1998). Cancer - awakening angels. *Nature* ***394***, 616-617.

Lane, D.P. (1992). Cancer - p53, guardian of the genome. *Nature* ***358***, 15-16.

Lanza, G., Gafa, R., Santini, A., Maestri, I., Dubini, A., Gilli, G., and Cavazzini, L. (1998). Prognostic significance of DNA ploidy in patients with stage II and stage III colon carcinoma - a prospective flow cytometric study. *Cancer* ***82***, 49-59.

Larramendy, M.L., Tarkkanen, M., Valle, J., Kivioja, A.H., Ervasti, H., Karaharju, E., Salmivalli, T., Elomaa, I., and Knuutila, S. (1997). Gains, losses, and amplifications of DNA sequences evaluated by comparative genomic hybridization in chondrosarcomas. *American Journal of Pathology* ***150***, 685-691.

Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystromlahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach, R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, H., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. (1993). Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* ***75***, 1215-1225.

Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995). P53 and its 14 KDA c-terminal domain recognize primary DNA damage in the form of insertion deletion mismatches. *Cell* ***81***, 1013-1020.

Leister, I., Weith, A., Bruderlein, S., Cziepluch, C., Kangwanpong, D., Schlag, P., and Schwab, M. (1990). Human colorectal cancer - high frequency of deletions at chromosome 1p35. *Cancer Research* ***50***, 7232-7235.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1997a). DNA methylation and genetic instability in colorectal cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* ***94***, 2545-2550.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1997b). Genetic instability in colorectal cancers. *Nature* ***386***, 623-627.

Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature* ***396***, 643-649.

Leppert, M., Dobbs, M., Scambler, P., O'Connell, P., Nakamura, Y., Stauffer, D., Woodward, S., Burt, R., Hughes, J. and Gardner, E. (1987). The gene for familial polyposis coli maps to the long arm of chromosome 5. *Science* ***238***, 1411-1413.

Levine, A.J., Chang, A.W., Dittmer, D., Notterman, D.A., Silver, A., Thorn, K., Welsh, D., and Wu, M. (1994). The p53 tumor suppressor gene. *Journal of Laboratory and Clinical Medicine* ***123***, 817-823.

Levine, A.J., Momand, J., and Finlay, C.A. (1991). The p53 tumor suppressor gene. *Nature* ***351***, 453-456.

Li, Y. and Benezra, R. (1996). Identification of a human mitotic checkpoint gene: hsMAD2. *Science* ***274***, 246-248.

Liggett, W.H. and Sidransky, D. (1998). Role of the p16 tumor suppressor gene in cancer. *Journal of Clinical Oncology* ***16***, 1197-1206.

Limoli, C.L., Kaplan, M.I., Corcoran, J., Meyers, M., Boothman, D.A., and Morgan, W.F. (1997). Chromosomal instability and its relationship to other end points of genomic instability. *Cancer Research* ***57***, 5557-5563.

Lin, H.Y., Wang, X-F., Ng-Eaton, E., Weinberg, R.A. and Lodish, H.F. (1992). Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. *Cell* ***68***, 775-785.

Liu, L., Markowitz, S. and Gerson, S.L. (1996a). Mismatch repair mutations ovrride alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Research* ***56***, 18183-18186.

Liu, B., Nicolaides, N.C., Markowitz, S., Willson, J.K.V., Parsons, R.E., Jen, J., Papadopolous, N., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. (1995). Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genetics* ***9***, 48-55.

Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1996b). Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Medicine* ***2***, 169-174.

Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T.D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* ***70***, 923-935.

Loeb, L.A. (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Research* ***51***, 3075-3079.

Losi, L., Roncucci, L., di Gregorio, C., de Leon, M.P. and Benhattar, J. (1996). K-ras and p53 mutations in human colorectal aberrant crypt foci. *Journal of Pathology* ***178***, 259-263.

Lothe, R.A., Peltomaki, P., Meling, G.I., Aaltonen, L.A., Nystrom-Lahti, M., Pylkkanen, L., Heimdal, K., Andersen, T.I., Moller, P., Rognum, T.O., Fossa, S.D., Haldorsen, T., Langmark, F., Brogger, A., de la Chapelle, A., and Borresen, A.L. (1993). Genomic instability in colorectal cancer - relationship to clinicopathological variables and family history. *Cancer Research* ***53***, 5849-5852.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993a). P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* ***74***, 957-967.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. (1993b). P53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* ***362***, 847-849.

Lu, S-L., Zhang, W-C., Akiyama, Y., Nomizu, T. and Yuasa, Y. (1996). Genomic structure of the transforming growth factor β type II receptor gene and its mutations in Hereditary Nonpolyposis Colorectal Cancers. *Cancer Research* ***56***, 4595-4598.

Lucas, K.G., Pollok, K.E., and Emanuel, D.J. (1997). Post-transplant EBV induced lymphoproliferative disorders. *Leukemia & Lymphoma* ***25***, 1-8.

Lukish, J.R., Muro, K., De Nobile, J., Katz, R., Williams, J., Cruess, D.F., Drucker, W., Kirsch, I., and Hamilton, S.R. (1998). Prognostic significance of DNA replication errors in young patients with colorectal cancer. *Annals of Surgery* ***227***, 51-56.

MacPhee, M., Chepenik, K.P., Liddell, R.A., Nelson, K.K., Siracusa, L.D., and Buchberg, A.M. (1995). The secretory phospholipase-A2 gene is a candidate for the MOM1 locus, a major modifier of APC(MIN)-induced intestinal neoplasia. *Cell* ***81***, 957-966.

Maekawa, M., Sugano, K., Kashiwabara, H., Ushiama, M., Fujita, S., Toshimori, M. and Kakizoe, T. (1999). DNA methylation analysis using bisulfite treatment and PCR-single-strand conformation polymorphism in colorectal cancer showing microsatellite instability. *Biochemical and Biophysical Research Communications* ***262***, 671-676.

Mahlamaki, E.H., Hoglund, M., Gorunova, L., Karhu, R., Dawiskiba, S., Andrensandberg, A., Kallioniemi, O.P., and Johansson, B. (1997). Comparative genomic hybridization reveals frequent gains of 20q, 8q, 11q, 12q, and 17q, and losses of 18q, 9p, and 15q in pancreatic cancer. *Genes Chromosomes & Cancer* ***20***, 383-391.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L.Z., Lutterbaugh, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Vogelstein, B., Brattain, M., and Willson, J.K.V. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* ***268***, 1336-1338.

Marsischky, G.T., Filosi, N., Kane, M.F., and Kolodner, R. (1996). Redundancy of saccharomyces cerevisiae MSH3 and msh6 in MSH2-dependent mismatch repair. *Genes & Development* ***10***, 407-420.

Massague, J., Hata, A. and Liu, F. (1997). TGF-β signalling through the Smad pathway. *Trends in Cell Biology 7,* 187-192.

Mathew, L.S. and Vale, W.W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* ***65***, 973-982.

Matsuzaki, M., Nagase, S., Abe, T., Miura, K., Shiiba, K., Sunamura, M., and Horii, A. (1998). Detailed deletion mapping on chromosome 1p32-p36 in human colorectal cancer: identification of three distinct regions of common allelic loss. *International Journal of Oncology* ***13***, 1229-1233.

Melhem, M.F., Meisler, A.I., Finley, G.G., Bryce, W.H., Jones, M.O., Tribby, I.I., Pipas, J.M. and Koski, R.A. (1992). Distribution of cells expressing myc proteins in human colorectal epithelium, polyps, and malignant tumors. *Cancer Research* ***52***, 5853-5864.

Meling, G.I., Lothe, R.A., Borresen, A.L., Graue, C., Hauge, S., Clausen, O.P.F., and Rognum, T.O. (1993). The tp53 tumor suppressor gene in colorectal carcinomas . Relation to DNA ploidy pattern and clinicopathological variables. *British Journal of Cancer* ***67***, 93-98.

Meling, G.I., Lothe, R.A., Borresen, A.L., Graue, C., Hauge, S., Clausen, O.P.F., and Rognum, T.O. (1993). The tp53 tumor suppressor gene in colorectal carcinomas .1. Genetic alterations on chromosome 17. *British Journal of Cancer* ***67***, 88-92.

Mello, J.A., Acharya, S., Fishel, R., and Essigmann, J.M. (1996). The mismatch repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. *Chemistry & Biology* ***3***, 579-589.

Mertens, F., Johansson, B., Hoglund, M., and Mitelman, F. (1997). Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. *Cancer Research* ***57***, 2765-2780.

Messerini, L., Ciantelli, M., Baglioni, S., Palomba, A., Zampi, G. and Papi, L. (1999). Prognostic significance of microsatellite instability in sporadic mucinous colorectal cancers. *Human Pathology* ***30***, 629-634.

Messerini, L., Vitelli, F., De Vitis, L.R., Mori, S., Calzolari, A., Palmirotta, R., Calabr`o, A. and Papi, L. (1997). Microsatellite instability in sporadic mucinous colorectal carcinomas: relationship to clinico-pathological variables. *Journal of Pathology* ***182***, 380-384.

Milner, J. and Medcalf, E.A. (1991). Cotranslation of activated mutant p53 with wild-type drives the wild-type p53 protein into the mutant conformation. *Cell* ***65***, 765-774.

Mitelman, F., Mertens, F., and Johansson, B. (1997). A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nature Genetics* ***15***, 417-474.

Miyaki, M., Konishi, M., Tanaka, K., KikuchiYanoshita, R., Muraoka, M., Yasuno, M., Igari, T., Koike, M., Chiba, M., and Mori, T. (1997). Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. *Nature Genetics* ***17***, 271-272.

Miyashiro, I., Senda, T., Matsumine, A., Baeg, G.H., Kuroda, T., Shimano, T., Miura, S., Noda, T., Kobayashi, S. and Monden, M. (1995). Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin. *Oncogene* ***11***, 89-96.

Miyashita, T. and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* ***80***, 293-299.

Miyazaki, M., Furuya, T., Shiraki, A., Sato, T., Oga, A. and Sasaki, K. (1999). The relationship of DNA ploidy to chromosomal instability in primary human colorectal cancers. *Cancer Research* ***59***, 5283-5285.

Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T. and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumours: mutation cluster region in the APC gene. *Human Molecular Genetics* ***1***, 229-233.

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. *Annual Review of Genetics* ***25***, 229-253.

Modrich, P. (1995). Mismatch repair, genetic stability and tumour avoidance. *Philosophical Transactions of the Royal Society of London. series B: Biological Sciences* ***347***, 89-95.

Modrich, P. and Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annual Review of Biochemistry* ***65***, 101-133.

Monk, M. (1995). Epigenetic programming of differential gene expression in development and evolution. *Developmental Genetics* ***17***, 188-197.

Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1996). Apoptosis and APC in colorectal tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* ***93***, 7950-7954.

Moser, A.R., Pitot, H.C., and Dove, W.F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* ***247***, 322-324.

Mosier, D.E., Baird, S.M., Kirven, M.B., Gulizia, R.J., Wilson, D.B., Kubayashi, R., Picchio, G., Garnier, J.L., Sullivan, J.L., and Kipps, T.J. (1990). EBV-associated B-cell lymphomas following transfer of human peripheral blood lymphocytes to mice with severe combined immune deficiency. *Current Topics in Microbiology dnd Immunology* ***166***, 317-323.

Motwani, B.T., Shafir, M., Merric, M., Tepper, J. and Bruckner, H.W. Adenocarcinoma of the colon and Rectum. In Pine, J.W. ed. *Cancer Medicine*. Baltimore: Williams and Wilkins. 1997, 2029-2072.

Muleris, M., Salmon, R.J., and Dutrillaux, B. (1988). Existence of 2 distinct processes of chromosomal evolution in near-diploid colorectal tumors. *Cancer Genetics dnd Cytogenetics* ***32***, 43-50.

Muleris, M., Salmon, R.J., and Dutrillaux, B. (1990). Cytogenetics of colorectal adenocarcinomas. *Cancer Genetics and Cytogenetics* ***46***, 143-156.

Muleris, M., Salmon, R.J., Zafrani, B., Girodet, J. and Dutrillaux, B. (1985). Consistent deficiencies of chromosome 18 and of the short arm of chromosome 17 in eleven cases of human large bowel cancer: a possible recessive determinism. *Annales de Genetique* ***28***, 206-213.

Muleris, M., Zafrani, B., Validire, P., Girodet, J., Salmon, R.J., and Dutrillaux, B. (1994). Cytogenetic study of 30 colorectal adenomas. *Cancer Genetics and Cytogenetics* ***74***, 104-108.

Murphy, W.J., Funakoshi, S., Beckwith, M., Rushing, S.E., Conley, D.K., Armitage, R.J., Fanslow, W.C., Rager, H.C., Taub, D.D., Ruscetti, F.W., and Longo, D.L. (1995). Antibodies to CD40 prevent Epstein-Barr virus-mediated human B-cell lymphomagenesis in severe combined immune-deficient mice given human peripheral blood lymphocytes. *Blood* ***86***, 1946-1953.

Nalesnik, M.A. (1998). Clinical and pathological features of post-transplant lymphoproliferative disorders (PTLD). *Springer Seminars in Immunopathology* ***20***, 325-342.

Nelson, W.G. and Kastan, M.B. (1994). DNA strand breaks - the DNA template alterations that trigger p53- dependent DNA damage response pathways. *Molecular and Cellular Biology* ***14***, 1815-1823.

Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and Kinzler, K.W. (1994). Mutations of 2 PMS homologs in hereditary nonpolyposis colon cancer. *Nature* ***371***, 75-80.

Nigg, E.A. (1998). Polo-like kinases: positive regulators of cell division from start to finish. *Current Opinion in Cell Biology* ***10***, 776-783.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modali, R., Harris, C.C., and Vogelstein, B. (1989). Mutations in the p53 gene occur in diverse human tumor types. *Nature* ***342***, 705-708.

Nishino, H., Knoll, A., Buettner, V.L., Frisk, C.S., Maruta, Y., Haavik, J., and Sommer, S.S. (1995). P53 wild-type and p53 nullizygous big blue transgenic mice have similar frequencies and patterns of observed mutation in liver, spleen and brain. *Oncogene* ***11***, 263-270.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S. and Hedge, P. (1991). Mutations of chromosome 5q21 in FAP and colorectal cancer patients. *Science* ***253***, 665-669.

Nowell, P.C. (1976). The clonal evolution of tumor cell populations. Acquired genetic lability permits stepwise selection of variant sublines and underlies tumor progression. *Science* ***194***, 23-28.

Nystrom-Lahti, M., Parsons, R., Sistonen, P., Pylkkanen, L., Aaltonen, L.A., Leach, F.S., Hamilton, S.R., Watson, P., Bronson, E., Cavalieri, J., Lynch, J., Lanspa, S., Smyrk, T., Lynch, P., Drouhard, T., Kinzler, K.W., Vogelstein, B., Lynch, H.T., de la Chapelle, A., and Peltomaki, P. (1994). Mismatch repair genes on chromosomes 2p and 3p account for a major share of hereditary nonpolyposis colorectal cancer families evaluable by linkage. *American Journal of Human Genetics* ***55***, 659-665.

Ogunbiyi, O.A., Goodfellow, P.J., Gagliardi, P.E., Swanson, P.E, Birnbaum, E.H., Fleshman, J.W., Kodner, I.J. and Moley, J.F. (1997). Prognostic value of chromosome 1p allelic loss in colon cancer. *Gastroenterology* ***113***, 761-766.

Olschwang, S., Hamelin, R., Laurent-Puig, P., Thuille, B., De Rycke, Y., Li, Y.J., Muzeau, F., Girodet, J., Salmon, R.J. and Thomas, G. (1997). Alternative genetic pathways in colorectal carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* ***94***, 12122-12127.

Oren, M. (1985). The p53 cellular tumor antigen - gene structure, expression and protein properties. *Biochimica et Biophisica Acta* ***823***, 67-78.

Ottini, L., Palli, D., Falchetti, M., Damico, C., Amorosi, A., Saieva, C., Calzolari, A., Cimoli, F., Tatarelli, C., De Marchis, L., Masala, G., Mariani-Costantini, R., and Cama, A. (1997). Microsatellite instability in gastric cancer is associated with tumor location and family history in a high risk population from tuscany. *Cancer Research* ***57***, 4523-4529.

Ouyang, H., Furukawa, T., Abe,T., Kato,Y. and Horii, A. (1998). The BAX gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach, and endometrium. *Clinical Cancer Research* ***4***, 1071-1074.

Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Ruben, S.M., Carter, K.C., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Hamilton, S.R., Petersen, G.M., Watson, P., Lynch, H.T., Peltomaki, P., Mecklin, J.P., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. (1994). Mutation of a mutl homolog in hereditary colon cancer. *Science* ***263***, 1625-1629.

Parsons, R., Myeroff, L.L., Liu, B., Willson, K.V., Markowitz, S.D., Kinzler, K.W. and Vogelstein B. (1995). Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. *Cancer Research* ***55***, 320-324.

Paulovich, A.G., Toczyski, D.P and Hartwell, L.H. (1997). When checkpoints fail. *Cell* ***88***, 315-321.

Peltomaki, P., Lothe, R.A., Aaltonen, L.A., Pylkkanen, L., Nystromlahti, M., Seruca, R., David, L., Holm, R., Ryberg, D., Haugen, A., Brogger, A., Borresen, A.L., and de la Chapelle, A. (1993). Microsatellite instability is associated with tumours that characterize the hereditary nonpolyposis colorectal carcinoma syndrome. *Cancer Research* ***53***, 5853-5855.

Pisani, P., Parkin, D.M., Munoz, N., and Ferlay, J. (1997). Cancer and infection: estimates of the attributable fraction in 1990. *Cancer Epidemiology Biomarkers & Prevention* ***6***, 387-400.

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B. and Kinzler, K.W. (1992). APC mutations occur early during colorectal tumorigenesis. *Nature* ***359***, 235-237.

Praml, C., Finke, L.H., Herfarth, C., Schlag, P., Schwab, M., and Amler, L. (1995). Deletion mapping defines different regions in 1p34.2-pter that may harbor genetic information related to human colorectal cancer. *Oncogene* ***11***, 1357-1362.

Praml, C., Savelyeva, L., Perri, P. and Schwab, M. (1998). Cloning of the human aflatoxin B1-aldehyde reductase gene at 1p35-1p36.1 in a region frequently altered in human tumor cells. *Cancer Research* ***58***, 5014-5018.

Pretlow, T.P., Brasitus, T.A., Fulton, N.C., Cheyer, C. and Kaplan, E.L. (1993). K-ras mutations in putative preneoplastic lesions in human colon. *Journal of National Cancer Institute* ***85***, 2004-2007.

Purdie, C.A., Ogrady, J., Piris, J., Wyllie, A.H., and Bird, C.C. (1991). P53 expression in colorectal tumors. *American Journal of Pathology* ***138***, 807-813.

QuintanillaMartinez, L., Lome-Maldonado, C., Schwarzmann, F., Gredler, E., Reyes, E., Angeles-Angeles, A., and Fend, F. (1998). Post-transplantation lymphoproliferative disorders in Mexico: an aggressive clonal disease associated with Epstein-Barr virus type. *Modern Pathology* ***11***, 200-208.

Quirke, P., Dyson, J.E.D., Dixon, M.F., Bird, C.C., and Joslin, C.A.F. (1985). Heterogeneity of colorectal adenocarcinomas evaluated by flow-cytometry and histopathology. *British Journal of Cancer* ***51***, 99-106.

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C., and Perucho, M. (1997). Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* ***275***, 967-969.

Reichmann, A., Martin, P., and Levin, B. (1981). Chromosomes in human large bowel cancer - evidence for non-random changes. *Gastroenterology* ***80***, 1259

Remvikos, Y., Muleris, M., Salmon, R.J., and Dutrillaux, B. (1997). Colorectal carcinogenesis: from chromosomal evolution pathways to molecular pathogenesis. *Cancer Genetics and Cytogenetics* ***93***, 63-73.

Remvikos, Y., Muleris, M., Vielh, P., Salmon, R.J., and Dutrillaux, B. (1988). DNA content and genetic evolution of human colorectal adenocarcinoma - a study by flow-cytometry and cytogenetic analysis. *International Journal of Cancer* ***42***, 539-543.

Remvikos, Y., Vogt, N., Muleris, M., Salmon, R.J., Malfoy, B. and Dutrillaux, B. (1995). DNA-repeat instability is associated with colorectal cancers presenting minimal chromosome rearrangements. *Genes Chromosomes Cancer* ***12***, 272-276.

Resta, N., Simone, C., Mareni, C., Montera, M., Gentile, M., Susca, F., Gristina, R., Pozzi, S., Bertario, L., Bufo, P., Carlomagno, N., Ingrosso, M., Rossini, F.P., Tenconi, R., and Guanti, G. (1998). STK11 mutations in Peutz-Jeghers syndrome and sporadic colon cancer. *Cancer Research* ***58***, 4799-4801.

Reznikoff, C.A., Belair, C.D., Yeager, T.R., Savelieva, E., Blelloch, R.H., Puthenveettil, J.A., and Cuthill, S. (1996). A molecular genetic model of human bladder cancer pathogenesis. *Seminars in Oncology* ***23***, 571-584.

Rideout, W.M., Coetze, G.A., Olumni, A.F. and Jones, P.A. (1990). 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* ***249***, 1288-1290.

Ried, T., Knutzen, R., Steinbeck, R., Blegen, H., Schrock, E., Heselmeyer, K., du Manoir, S., and Auer, G. (1996). Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes & Cancer* ***15***, 234-245.

Risinger, J.I., Umar, A., Boyd, J., Berchuck, A., Kunkel, T.A. and Barrett, J.C. (1996). Mutation of MSH3 in endometrial cancer and evidence for its functional role in heteroduplex repair. *Nature Genetics* ***14***, 102-105.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B. (1985). Type β: transforming growth factor: a bifunctional regulator of cellular growth. *Proceedings of the National Academy of Sciences of the United States of America* ***82***: 119-123.

Rodrigues, N.R., Rowan, A., Smith, M.E.F., Kerr, I.B., Bodmer, W.F., Gannon, J.V., and Lane, D.P. (1990). P53 mutations in colorectal cancer. *Proceedings of the National Academy of Sciences of the United States of America* ***87***, 7555-7559.

Rosai, J. Gastrointerstinal Tract. In Rosai, J. ed. *Ackerman's Surgical Pathology*. St. Luis: Mosby. 1996, 589-814.

Rotman, G. and Shiloh, Y. (1998). ATM: from gene to function. *Human Molecular Genetics* **7**, 1555-1563.

Rowe, M., Young, L.S., and Rickinson, A.B. (1990). Analysis of Epstein-Barr virus gene expression in lymphomas derived from normal human B-cells grafted into scid mice. *Current Topics in Microbiology and Immunology* **166**, 325-331.

Royds, J.A., Sharrard, R.M., Wagner, B. and Polacarz, S.V. (1992). Cellular localisation of c-myc product in human colorectal epithelial neoplasia. *Journal of Pathology* **166**, 225-233.

Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S.H., Masiarz, F.R, Munemitsu, S. and Polakis, P. (1993). Association of the APC gene product with beta-catenin. *Science* **262**, 1731-1734.

Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G. and Lindblom, A. (1999). Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer* **26**, 247-252.

Sands, A.T., Suraokar, M.B., Sanchez, A., Marth, J.E., Donehower, L.A., and Bradley, A. (1995). P53 deficiency does not affect the accumulation of point mutations in a transgene target. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8517-8521.

Scherer, S.J., Welter, C., Zang, K.D., and Dooley, S. (1996). Specific in vitro binding of p53 to the promoter region of the human mismatch repair gene hMSH2. *Biochemical and Biophysical Research Communications* **221**, 722-728.

Schlegel, J., Stumm, G., Scherthan, H., Bocker, T., Zirngibl, H., Ruschoff, J., and Hofstadter, F. (1995). Comparative genomic *in situ* hybridization of colon carcinomas with replication error. *Cancer Research* **55**, 6002-6005.

Schutte, M., Hruban, R.H., Hedrick, L., Cho, K.R., Nadasdy, G.M., Weinstein, C.L., Bova, G.S., Isaacs, W.B., Cairns, P., Nawroz, H., Sidransky, D., Casero, R.A., Meltzer, P.S., Hahn, S.A., and Kern, S.E. (1996). DPC4 gene in various tumor types. *Cancer Research* **56**, 2527-2530.

Scott, N., Bell, S.M., Sagar, P., Blair, G.E., Dixon, M.F., and Quirke, P. (1993). P53 expression and K-ras mutation in colorectal adenomas. *Gut* **34**, 621-624.

Screaton, G.R., Bell, M.V., Jackson, D.G., Cornelis, F.B., Gerth, U. and Bell, J.I. (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proceedings of the National Academy of Sciences of the United States of America 89*, 12160-12164.

Shackney, S.E. and Shankey, T.V. (1997). Common patterns of genetic evolution in human solid tumours. *Cytometry* **29**, 1-27.

Shibata, D., Peinado, M.A., Inov, Y., Malkhosyan, S. and Peruch, M. (1994). Genomic instability in repeated sequences is an early event in colorectal tumorigenesis that persists after transformation. *Nature Genetics* **6**, 273-281.

Sen, S., Zhou, H.Y., and White, R.A. (1997). A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. *Oncogene* **14**, 2195-2200.

Senba, S., Konishi, F., Okamoto, T., Kashiwagi, H., Kanazawa, K., Miyaki, M., Konishi, M., and Tsukamoto, T. (1998). Clinicopathologic and genetic features of nonfamilial colorectal carcinomas with DNA replication errors. *Cancer* ***82***, 279-285.

Shibata, D., Peinado, M.A., Ionov, Y., Malkhosyan, S., and Perucho, M. (1994). Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. *Nature Genetics* ***6***, 273-281.

Simms, L.A., RadfordSmith, G., Biden, K.G., Buttenshaw, R., Cummings, M., Jass, J.R., Young, J., Meltzer, S.J., and Leggett, B.A. (1998). Reciprocal relationship between the tumor suppressors p53 and BAX in primary colorectal cancers. *Oncogene* ***17***, 2003-2008.

Smith, A.J., Stern, H.S., Penner, M., Hay, K., Mitri, A., Bapat, B.V. and Gallinger, S. (1994). Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. *Cancer Research* ***54***, 5527-5530.

Smith, K.J, Johnson, K.A., Bryan, T.M., Hill, D.E., Markowitz, S., Willson, J.K., Paraskeva, C., Petersen, G.M., Hamilton, S.R. and Vogelstein, B. (1993). The APC gene product in normal and tumor cells. *Proceedings of the National Academy of Sciences of the United States of America* ***90***, 2846-2850.

Smith, L., Liu, S.J., Goodrich, L., Jacobson, D., Degnin, C., Bentley, N., Carr, A., Flaggs, G., Keegan, K., Hoekstra, M., and Thayer, M.J. (1998). Duplication of ATR inhibits MYOD, induces aneuploidy and eliminates radiation-induced G1 arrest. *Nature Genetics* ***19***, 39-46.

Solomon, E., Voss, R., Hall, V., Bodmer, W.F., Jass, J.R., Jeffreys, A.J., Lucibello, F.C., Patel, I. and Rider, S.H. (1987). Chromosome 5 allele loss in human colorectal carcinomas. *Nature* ***328***, 616-619.

Stamenkovic, I., Amiot, M., Pesando, J.M. and Seed, B. (1989). A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell* ***56***, 1057-1062.

Steinbeck, R.G. (1998). Chromosome division figures reveal genomic instability in tumorigenesis of human colon mucosa. *British Journal of Cancer 77*, 1027-1033.

Steinbeck, R.G., Heselmeyer, K.M., and Auer, G.U. (1994). DNA ploidy in human colorectal adenomas. *Analytical and Quantitative Cytology and Histology* ***16***, 196-202.

Strauss, B.S. (1998). Hypermutability in carcinogenesis. *Genetics* ***148***, 1619-1626.

Su, L.K., Burell, M., Hill, D.E., Gyuris, J., Brent, R., Wiltshire, R., Trent, J., Vogelstein, B. and Kinzler, K.W. (1995). APC binds to the novel protein EB1. *Cancer Research* ***55***, 2972-2977.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A. and Dove, W.F. (1992). Multiple interstinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* ***256***, 668-670.

Su, L.K., Vogelstein, B. and Kinzler, K.W. (1993). Association of the APC tumor suppressor protein with catenins. *Science 262*, 1734-1737.

Takagi, Y., Kohmura, H., Futamura, M., Kida, H., Tanemura, H., Shimokawa, K., and Saji, S. (1996). Somatic alterations of the DPC4 gene in human colorectal cancers *in vivo*. *Gastroenterology* ***111***, 1369-1372.

Thiagalingam, S., Lengauer, C., Leach, F.S., Schutte, M., Hahn, S.A., Overhauser, J., Willson, J.K.V., Markowitz, S., Hamilton, S.R., Kern, S.E., Kinzler, K.W., and Vogelstein, B. (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nature Genetics* ***13***, 343-346.

Thibodeau, S.N., Bren, G., and Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science* ***260***, 816-819.

Thibodeau, S.N., French, A.J., Cunningham, J.M., Tester, D., Burgart, L.J., Roche, P.C., McDonnell, S.K., Schaid, D.J., Vockley, C.W., Michels, V.V., Farr, G.H., and O'Connell, M.J. (1998). Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Research* ***58***, 1713-1718.

Togo, G., Toda, N., Kanai, F., Kato, N., Shiratori, Y., Kishi, K., Imazeki, F., Makuuchi, M., and Omata, M. (1996a). A transforming growth factor beta type II receptor gene mutation common in sporadic cecum cancer with microsatellite instability. *Cancer Research* ***56***, 5620-5623.

Togo, G., Toda, N., Kanai, F., Kato, N., Yoshida, H., Shiratori, Y., and Omata, M. (1996b). Mutation of TGF-beta type II receptor gene in gastrointestinal and hepatobiliary carcinomas. *Gastroenterology* ***110***, A604

Tomlinson, I., Ilyas, M., and Novelli, M. (1997). Molecular genetics of colon cancer. *Cancer and Metastasis Reviews* ***16***, 67-79.

Tomlinson, I.P.M., Novelli, M.R., and Bodmer, W.F. (1996). The mutation rate and cancer. *Proceedings of the National Academy of Sciences of the United States of America* ***93***, 14800-14803.

Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B. and Issa, J.P. (1999). CpG island methylator phenotype in colorectal cancer. *Proceedings of the National Academy of Sciences of the United States of America* ***96***, 8681-8686.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C.M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* ***229***, 1390-1393.

Umar, A., Koi, M., Risinger, J.I., Glaab, W.E., Tindall, K.R., Kolodner, R.D., Boland, C.R., Barrett, J.C. and Kunkel, T.A. (1997). Correction of hypermutability, *N*-methyl-*N*-nitro-*N*-nitrosoguanindine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human tumor cells with mutations in MSH2, and MSH6. *Cancer Research* ***75***, 3949-3955.

Umar, A., Risinger, J.I., Glaab, W.E., Tindall, K.R., Barrett, J.C. and Kunkel, T.A. (1998). Functional overlap in mismatch repair by human MSH3 and MSH6. *Genetics* ***148***, 1637-1646.

Vandenbroek, M.H., Renault, B., Fodde, R., Verspaget, H., Griffioen, G., and Khan, P.M. (1993). Sites and types of p53 mutations in an unselected series of colorectal cancers in the Netherlands. *Anticancer Research* ***13***, 587-592.

Vasen, H.F., Mecklin, J.P., Khan, P.M. and Lynch, H.T. (1991). The International Collabotative Group on Hereditary Non-Polyposis Coloractal Cancer (ICG-HNPCC). *Diseases of the Colon and Rectum* ***34***, 424-425.

Venkatachalam, S., Shi, Y.P., Jones, S.N., Vogel, H., Bradley, A., Pinkel, D., and Donehower, L.A. (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *Embo Journal* ***17***, 4657-4667.

Verma, R.S. and Babu, A. Fluorescence in situ hybridisation techniques. In *Human Chromosomes: principles and techniques*. New York: McGraw-Hill, Inc., 1995, 191-209.

Vindelov, L.L., Christensen, I.J., and Nissen, N.I. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* ***3***, 323-327.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M.M., and Bos, J.L. (1988). Genetic alterations during colorectal tumor development. *New England Journal of Medicine* ***319***, 525-532.

Waldman, T., Zhang, Y., Dillehay, L., Yu, J., Kinzler, K., Vogelstein, B. and Williams, J. (1997). Cell-cycle arrest versus cell death in cancer therapy. *Nature Medicine* ***3***, 1034-1036.

Wang, C., Eshleman, J., Willson, J., Markowitz, S. TGF-β and substrate release are both inducers of apoptosis in a human colon adenoma cell line. (1995). *Cancer Research* ***55***, 5101-5105.

Watson, A.J., Merritt, A.J., Jones, L.S., Askew, J.N., Anderson, E., Becciolini, A., Balzi, M., Potten, C.S. and Hickman, J.A. (1996). Evidence of reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. *British Journal of Cancer* ***73***, 889-95.

Webb, S.J., Harrison, D.J. and Wyllie, A.H. (1997). Apoptosis: an overview of the process and its relevance in disease. *Advances in Pharmacology* ***41***, 1-34.

Weber, J.L. and May, P.E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* ***44***, 388-396.

Weber, J.L. and May, P.E. (1989). Polymorphisms based on length variations in blocks of (DC-DA).(DG-DT) repeats. *Cytogenetics and Cell Genetics* ***51***, 1103-1104.

Wheeler, J.M., Beck, N.E., Kim, H.C., Tomlinson, I.P., Mortensen, N.J. and Bodmer, W.F. (1999). Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of hMLH1. *Proceedings of the National Academy of Sciences of the United States of America* ***96***, 10296-10301.

Whitehouse, A., Meredith, D.M., and Markham, A.F. (1998). DNA mismatch repair genes and their association with colorectal cancer (review). *International Journal of Molecular Medicine* ***1***, 469-474.

Wielenga, V.J., van der Neut, R., Offerhaus, G.J. and Pals, S.T. (2000). CD44 glycoproteins in colorectal cancer: expression, function, and prognostic value. *Advances in Cancer Research 77*, 169-187.

Wijnen, J., Vasen, H., Khan, P.M., Menko, F.H., van der Klift, H., Van Leeuwen, C., Van den Broek, M., Van Leeuwen-Cornelisse, I., Nagengast, F., Meijersheijboer, A., Lindhout, D., Griffioen, G., Cats, A., Kleibeuker, J., Varesco, L., Bertario, L., Bisgaard, M.L., Mohr, J., and Fodde, R. (1995). 7 new mutations in hMSH2, an HNPCC gene, identified by denaturing gradient gel electrophoresis. *American Journal of Human Genetics* ***56***, 1060-1066.

Wood, B.L., Sabath, D., Broudy, V.C., and Raghu, G. (1996). The recipient origin of posttransplant lymphoproliferative disorders in pulmonary transplant patients - a report of three cases. *Cancer* ***78***, 2223-2228.

Wu, C., Akiyama, Y., Imai, K., Miyake, S., Nagasaki, H., Oto, M., Okabe, S., Iwama, T., Mitamura, K., Masumitsu, H., Nomizu, T., Baba, S., Maruyama, K., and Yuasa, Y. (1994). DNA alterations in cells from hereditary nonpolyposis colorectal cancer patients. *Oncogene* ***9***, 991-994.

Xiao, S., Wei, W., Feng, X.L., Shi, Y.H., Liu, Q.Z., and Li, P. (1992). Direct chromosome analysis of 7 primary colorectal carcinomas. *Cancer Genetics and Cytogenetics* ***62***, 32-39.

Yagi, O.K., Akiyama, Y., Nomizu, T., Iwama, T., Endo, M. and Yuasa, Y. (1998). Proapoptotic gene BAX is frequently mutated in nonpolyposis colorectal cancers but not in adenomas. *Gastroenterology* ***114***, 268-274.

Yamamoto, H., Itoh, F., Kusano, M., Yoshida, Y., Hinoda, Y. and Imai, K. (1998a). Infrequent inactivation of DCC gene in replication error-positive colorectal cancers. *Biochemical and Biophysiscal Research Communications* ***244***, 204-209.

Yamamoto, H., Sawai, H,m Weber, T.K., Rodriguez-Bigas, M.A. and Perucho, M. (1998b). Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. *Cancer Research* ***58***, 997-1003.

Yaremko, M.L., Wasylyshyn, M.L., Paulus, K.L., Michelassi, F., and Westbrook, C.A. (1994). Deletion mapping reveals 2 regions of chromosome 8 allele loss in colorectal carcinomas. *Genes Chromosomes & Cancer* ***10***, 1-6.

Yaseen, N.Y., Watmore, A.E., Potter, A.M., Potter, C.W., Jacob, G. and Rees, R.C. (1990). Chromosome studies in eleven colorectal tumours. *Cancer Genetics and Ctyogenetics* ***44***, 83-97.

Yin, Y.X., Tainsky, M.A., Bischoff, F.Z., Strong, L.C., and Wahl, G.M. (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* ***70***, 937-948.

Yonemoto, W., Garrod, S.M., Bell, S.M., and Taylor, S.S. (1993). Identification of phosphorylation sites in the recombinant catalytic subunit of cAMP-dependent protein kinase. *Journal of Biological Chemistry* ***268***, 18626-18632.

Zhou, H.Y., Kuang, J., Zhong, L., Kuo, W.L., Gray, J.W., Sahin, A., Brinkley, B.R., and Sen, S. (1998). Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nature Genetics* ***20***, 189-193.

Appendix 1a. Details of patients and tumours included in this study.

1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1n	M	55	as.c	r	C	2 of 8	nm								d					n	0							
1a							ac	md	no	-	-	-	-	-	nt	1.82	8	4	4	n	0		-	-	-	-	-	n
1b							ac	md	no	-	-	-	-	-	nt	1.84	14	8	6	n	0						+	n*
1c							ac	md	no	-	-	-	-	-	nt	1.84	20	13	7	n	0						+	n*
1d							ac	md	no	-	-	-	-	-	nt	1.82	6	2	4	n	0						-	n
2n	F	62	r	l	B		nm								d					n	0							
2a							ac	md	no	-	-	-	-	-	nd	1.1	12	2	10	p	80	intens.					-	d
2b							ac	md	no	-	-	-	-	-	nt	2.2	28	11	17	p	40	intens.					-	d
2c							ac	md	no	-	-	-	-	-	nd/nt	1.2/2.2	8	1	7	p	20	intens.					-	d
2d							ac	md	no	-	-	-	-	-	nd	1.1	16	10	6	p	80	intens.					-	d
3n	M	64	as.c	r	A		nm								d					n	0		-	-	-	-		
3a							ac	md	no	+	s	-	s	s	d		0	0	0	n	0		-	-	-	-	-	n
3b							ac	md	no	+	s	-	s	s	d		0	0	0	n	0		-	-	-	-	-	n
3c							ac	md	no	+	s	-	s	s	d		1	0	1	n	0		-	-	-	-	-	n
3d							ac	md	no	+	s	-	s	s	d		2	1	1	n	0		-	-	-	-	-	n
4n	F	72	r	l	B		nm								d					n	0							
4a							ac	md	no	-	-	-	-	-	a	1.54/3.05	13	5	8	n*	40	weak					-	d
4b							ac	md	no	-	-	-	-	-	a	1.6/3.13	11	4	7	n*	30	weak	mut	-	-	-	-	d
4c							ac	md	no	-	-	-	-	-	a	1.59/3.1	10	5	5	n*	10 to 90	weak					-	d
4d							ac	md	no	-	-	-	-	-	a	1.58/3.1	9	4	5	n*	30 to 80	weak					-	d
5n	M	81	s.c	l	B		nm								d					n	0							
5a							ac	md	no	-	-	-	-	-	a	1.65/3.2	14	5	9	n	0		-	pol.	-	-	-	n*
5b							ac	md	no	-	-	-	-	-	a	1.65/3.2	0	0	0	n	0			pol.			-	n*
5c							ac	md	no	-	-	-	-	-	a	1.58/3.08	2	1	1	n	0			pol.			-	n*
5d							ac	md	no	-	-	-	-	-	a	1.63	6	2	4	n	0						-	n*
6n	M	63	s.c	l	A		nm								d					n	0							
6a							ac	wd	no	-	-	-	-	-	a	1.66/3.22	10	5	5	p	10 to 100	intens.					-	d
6b							ac	wd	no	-	-	-	-	-	a	1.68/3.23	13	5	8	p	20 to 100	intens.					-	d
6c							ac	wd	no	-	-	-	-	-	a	1.72/3.33	11	5	6	p	10 to 90	intens.					-	d
6d							ac	wd	no	-	-	-	-	-	a	1.68/2.25/3.29	9	3	6	p	10 to 80	intens.					-	d

Appendix 1a. Details of patients and tumours included in this study.

7n	M	89	d.c	I	A		nm								d					n	0						
7a							ac	wd	no	-	-	-	-	-	nt	1.81/3.56	0	0	0	p	20	intens.					-	d
7b							ac	wd	no	-	-	-	-	-	a	1.76/3.49	7	1	6	p	10 to 100	intens.					-	d
7c							ac	wd	no	-	-	-	-	-	nt	1.85/3.55	0	0	0	p	20 to 100	intens.					-	d
8n	F	80	r	I	B		nm								d					n	0						
8a							ac	md	no	-	-	-	-	-	a	1.68/3.28	1	0	1	n	0		mut	-	-	-	-	d
8b							ac	md	no	-	-	-	-	-	a	1.73/3.37	10	2	8	n	0			-	-	-	+	d
8c							ac	md	no	-	-	-	-	-	a	1.76/3.42	8	5	3	n*	5	weak		-	-	-	+	d
8d							ac	md	no	-	-	-	-	-	a	1.65/1.96	9	5	4	n*	5	weak	mut	-	-	-	-	d
9n	F	59	r	I	C	9 of 15	nm								d					n	0						
9a							ac	pd	no	-	-	-	-	-	a	1.59/1.87	1	1	0	p	95	intens.					-	d
9b							ac	pd	no	-	-	-	-	-	nt	1.92	17	4	13	p	80	intens.					-	d
9c							ac	pd	no	-	-	-	-	-	nt	1.96	6	3	3	p	10 to 100	intens.					-	d
9d							ac	pd	no	-	-	-	-	-	nt	2.04	10	3	7	p	95	intens.					-	d
9a'							ac	pd	no						d		8	2	6	p	80	intens.					-	d
9b'							ac	pd	no						d		1	0	1	p	10 to 100	intens.					-	d
10n	M	57	r	I	A		nm								d					n	0						
10a							ac	md	no	-	-	-	-	-	d		2	0	2	n	0		-	-	-	-	-	n
10b							ac	md	no	-	-	-	-	-	d		8	1	7	n	0						-	n
10c							ac	md	no	-	-	-	-	-	d		4	0	4	n	0						-	n
11n	F	73	t.c	r	B		nm								d					n	0						
11a							ac	md	no	-	-	-	-	-	a	1.43/2.83	17	5	12	p	5 to 100	intens.					+	d
11b							ac	md	no	-	-	-	-	-	nt	1.05/4.05	11	4	7	p	5 to 90	intens.					+	d
11c							ac	md	no	-	-	-	-	-	a	1.45/2.8	25	11	14	p	5 to 90	intens.					+	d
11d							ac	md	no	-	-	-	-	-	a	1.36/2.63	17	6	11	p	95	intens.					-	d
12n	F	60	as.c	r	C	5 of 26	nm								d					n	0						
12a							ac	pd	yes	+	s	s	s	s	d		9	5	4	n*	10 to 30	weak	mut	-	-	-	-	d
12b							ac	pd	yes	+	s	s	s	s	d		7	1	6	n*	10 to 30	weak	mut				-	d
12c							ac	pd	yes	+	s	s	s	s	d		2	2	0	n*	10 to 30	weak	mut				-	d
12d							ac	pd	yes	+	s	s	s	s	d		9	5	4	n*	5 to 10	weak	-				+	?

Appendix 1a. Details of patients and tumours included in this study.

13n	M	77	r	l	C		nm								d					n	0						
13a							ac	md	no	-	-	-	-	-	d		5	1	4	n	0			-	-	-	-	n
13b							ac	md	no	-	-	-	-	-	d		2	1	1	n*	50 to 80	weak	-		-	-	-	n*
14n	M	46	r	l	C		nm								d					n	0						
14a							ac	md	no	-	-	-	-	-	a	1.15/1.94/2.2	16	8	8	n	0		-	-	-	-	+	n*
14b							ac	md	no	-	-	-	-	-	d		12	8	4	n	0		-	-	-	-	-	n
15n	F	70	r	l	B		nm								d					n	0						
15a							ac	md	no	-	-	-	-	-	a	1.4	3	0	3	n	0		-	-	-	-	+	n*
15b							ac	md	no	-	-	-	-	-	d		10	3	7	n	0		-	-	-	-	+	n*
16n	F	57	r	l	C		nm								d					n	0						
16a							ac	md	no	-	-	-	-	-	d		6	4	2	n	0			-	-	-	+	n*
16b							ac	md	no	-	-	-	-	-	d		6	6	0	n	0		-	-	-	-	-	n
17n	M	76	cae.	r	C	4 of 4	nm								d					n	0						
17a							ac	pd	no	+	-	s	s	s	d		5	2	3	n*	<1	weak	-	-	-	-	-	n
17b							ac	pd	no	+	-	s	s	s	d		5	2	3	n*	<1	weak					-	n
17c							ac	pd	no	+	s	s	s	s	d		7	3	4	n*	<1	weak					+	n*
17d							ac	pd	no	+	s	s	s	s	d		8	2	6	n*	<1	weak					+	n*
18n	F	77	as.c	r	B		nm								d					n	0						
18a							ac	pd	no	-	-	-	s	-	nt	1.93/3.92	9	3	6	n*	1 to 5	weak	-	-	-	-	-	n
18b							ac	pd	no	+	-	s	s	-	nt	1.95	4	1	3	n*	5 to 10	weak					-	n
18c							ac	pd	no	+	s	-	s	-	d		10	2	8	n*	10 to 30	weak					-	n
18d							ac	pd	no	-	-	-	s	-	nt	1.87	2	0	2	n*	5 to 10	weak					-	n
19n	M	49	r	l	C	2 of 20	nm								d					n	0						
19a							ac	md	no	-	-	-	-	-	a	1.54/1.98/3.02	5	1	4	n	0		-	-	-	-	-	?
19b							ac	md	no	-	-	-	-	-	a	1.54/3.03	15	4	11	n	0		-	mut	-	-	+	d
19c							ac	md	no	-	-	-	-	-	a	1.52/3.01	17	5	12	n	0			mut			+	d
20n	F	70	as.c	r	C	3 of 15	nm								d					n	0						
20a							ac	pd	yes	+	-	s	s	s	d		7	5	2	n	0		-	-	-	-	-	n
20b							ac	pd	yes	+	-	-	s	s	d		1	1	0	n	0						-	n
20c							ac	pd	yes	+	-	s	s	s	d		4	1	3	n	0						-	n
20d							ac	pd	yes	+	-	-	s	s	d		2	1	1	n	0						-	n

Appendix 1a. Details of patients and tumours included in this study.

22n	F	81	as.c	r	C	3 of 8	nm								d					n	0						
22a							ac	md	no	-	-	-	-	-	a	1.32/2.43	7	3	4	p	5 to 90	intens.	mut	-	-	-	-	d
22b							ac	md	no	-	-	-	-	-	a	1.32	3	2	1	p	5 to 100	intens.	mut				-	d
22c							ac	md	no	-	-	-	-	-	a	1.4	14	4	10	p	30	intens.	mut				+	d

Key to Appendix 1a.

Column headings:

1: Sample ID
2: UB no - Department of Pathology reference no
3: RL no - Department of Pathology research laboratory reference no
4: Patient's sex: F - female
M - male
5: Patient's age
6: Tumour site:cae. - caecum
as.c - ascending colon
t.c - transverse colon
d.c - descending colon
r - rectum
7:Tumour location : r - right bowel
l - left bowel
8:Dukes' stage
9: No of lymphnodes involved if known
10: Sample's histology: nm - normal mucosa
ac - adenocarcinoma
11: Differentiation: wd - well differentiated
md - moderately differentiated
pd - poorly differentiated
12: Evidence of mucin production
13: RER status
14: Locus D2S123: -, no shift detected
s, shift present
15: Locus D13S160: -, no shift detected
s, shift present
16:Bat-26: -, no shift detected
s, shift present
17:TGF-beta RII: -, no shift detected
s, shift present
18:Ploidy: d - diploid
nd - near diploid
a - aneuploid
nt - near tetraploid
19: DNA index
20: Total no of chromosomal changes as detected by CGH
21: No of chromosomal gains detected by CGH
22: No of chromosomal losses detected by CGH
23: p53 IHC result: n - negative
p - positive
24: p53 IHC - %of cells stained
25: Intensity of p53 IHC staining: intens. - intensive
26: p53 exon 5: -, no mutation detected by SSCP
mut. - mutation present by SCCP
27: p53 exon 6: -, no mutation detected by SSCP
mut. - mutation present by SSCP
pol. - polymorphism noticed by SSCP
28: p53 exon 7: -, no mutation detected by SSCP
mut. - mutation present by SSCP
29: p53 exon 8: -, no mutation detected by SSCP
mut. - mutation present by SSCP
30: 17p loss as detected by CGH:
- not detected
+ present
31: Combined p53 status:
n - p53 functional
n*-p53 probably functional
d - p53 defective
? - undetermined

Appendix 1b. Details of xenografts included in this study.

1	2	3	´ 4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1xa	CAROa	0-1	24.04.97	11.08.97	15.5	97/648	ac	p	+	-	-	-	-	-	nt	1.85/3.61	31	17	14	n			-	-	-	-	-	n
1xb	CAROb	0-1	24.04.97	27.06.97	9	97/522	ac	m	-	-	-	-	-	-	nt	1.89/3.7	33	18	15	n							-	n
1xc	CAROc	0-1	24.04.97	24.07.97	15.5	97/648	ac	m	-	-	-	-	-	-	nt	1.80/2.05/3.53	30	11	19	n							-	n
1xd	CAROd	0-1	24.04.97	11.09.97	20	97/647	ac	m	-	-	-	-	-	-	nt	1.84	16	10	6	n							-	n
3xb	ALSIb	0-1	7.05.97	24.10.97	24	97/720	lym	p	-	-	-	-	-	-	d		3	1	2	n*	<10	weak	-		-	-	-	n
4xb	BRVIb	0-1	5.05.97	10.12.97		97/955	ac	m	-	-	-	-	-	-	a	1.94	19	10	9	n*	>90	weak	mut	-	-	-	+	d
5xa	ANSCa	0-1	22.05.97	26.09.97	18	97/720	ac	m		-	-	-	-	-	a	1.75/3.42	22	5	17	n			-	loh	-	-	+	n*
5xb	ANSCb	0-1	22.05.97	24.07.97	9	97/648	ac	m		-	-	-	-	-	a	1.64/3.24	9	2	7	n				loh			-	n
5xc	ANSCc	0-1	22.05.97	24.07.97	9	97/648	ac	m		-	-	-	-	-	a	1.63/3.2	22	7	15	n				loh			-	n
8xb	TSSHb	0-1	7.05.97	24.10.97	24.5	97/840	ac	m		-	-	-	-	-	a	1.75/3.44				n*	30	weak	mut	-	-	-		d
12xa	CABAa	0-1	26.08.97	26.09.97	4	97/720	ac	p	+	+	s	s	s	s	d		13	5	8	n			mut	-	-	-	+	d
12xb	CABAb	0-1	26.08.97	19.09.97	3	97/720	ac	p	+	+	s	s	s	s	d		7	1	6	n*	<1	weak	mut				+	d
12xc	CABAc	0-1	26.08.97	17.10.97	7.5	97/764	ac	p	+	+	s	s	s	s	d		8	3	5	n*	10 to 40	weak	mut				-	d
12xd	CABAd	0-1	26.08.97	19.09.97	3	97/764	ac	p	-	+	s	s	s	s	d		12	5	7	n*	<10	weak	mut				+	d
18xa	LISTa	0-1	22.10.97	22.01.98	12.5	97/926	ac	m	-	+	s	s	s	s	nt	1.83	20	8	12	n			-	-	-	-	-	n
18xb	LISTb	0-1	22.10.97	22.01.98	12.5	97/926	ac	m	-	+	s	s	s	s	nt	1.93				n								n
18xc	LISTc	0-1	22.10.97	22.01.98	12.5	97/926	ac	m	-	+	s	s	s	s	d		6	1	5	n							-	n
18xd	LISTd	0-1	22.10.97	22.01.98	12.5	97/726	ac	m	-	+	s	s	s	s	nt	1.98				n								n
19xa	JOMAa	0-1	5.11.97	27.01.98	12	98/51	ac	p	+	+	s	s	s	s	nt	1.93	6	3	3	n			-	-	-	-	-	n
19xb	JOMAb	0-1	5.11.97	27.01.98	12	98/51	ac	m	-	-	-	-	-	-	a	1.62/3.2	27	10	17	n			-	mut	-	-	-	d
19xc	JOMAc	0-1	5.11.97	27.01.98	12	98/51	ac	m	-	-	-	-	-	-	a	1.63/3.25	25	9	16	n				mut			-	d
20xa	JOTHa	0-1	5.11.97	22.01.98	11	97/955	ac	p	-/+	+	s	s	s	s	d		11	2	9	n				-	-	-	-	n
20xc	JOTHc	0-1	5.11.97	22.01.98	11	97/955	ac	p	-/+	+	s	s	s	s	d		9	4	5	n							-	n
20xd	JOTHd	0-1	5.11.97	22.01.98	11	97/955	ac	p	+	+	s	-	s	s	d		7	3	4	n							-	n
22xa	HEKIa	0-1	18.11.97	22.01.98	9.5	98/138	ac	p	-/+	-	-	-	-	-	a	1.34/2.6	29	8	21	n*	20	weak		mut.	-	-	-	d
22xb	HEKIb	0-1	18.11.97	22.01.98	9.5	98/138	ac	m	-	-	-	s	-	-	a	1.8/3.48	23	6	17	n				mut.			+	d
22xc	HEKIc	0-1	18.11.97	22.01.98	9.5	98/138	ac	m	-	-	-	-	-	-	a	1.42/2.72	25	8	17	n*	10 to 20	weak		mut.			-	d

Key to Appendix 1b.

Column headings:

1: Xenograft's ID
2: Xenograft's name
3: Passage no
4: Date of xenograft implantation in SCIDmice
5: Date of harvesting xenograft tissue
6: Passage time
7: RL no - Department of Pathology research laboratory reference no
8: Xenograft's histology : ac - adenocarcinoma
lym - lymphoma
9: Differentiation: m - moderate
p - poor
10: Evidence of mucin production
11: RER status
12: Locus D2S123: -, no shift detected
s, shift present
13: Locus D13S160: -, no shift detected
s, shift present
14: Bat-26: -, no shift detected
s, shift present
15: TGF-beta RII: -, no shift detected
s, shift present
16: Ploidy: d - diploid
nd - near diploid
a - aneuploid
nt - near tetraploid
17: DNA index
18: Total no of chromosomal changes detected by CGH
19: No of chromosomal gains detected by CGH
20: No of chromosomal losses detected by CGH
21: p53 IHC result: n - negative
n*- weak staining or staining present in less than 10% if cells
p - positive
22: p53 IHC - %of cells stained
23: Intensity of p53 IHC staining: intens. - intensive
24: p53 exon 5: -, no mutation detected by SSCP
mut. - mutation present bySSCP
25: p53 exon 6: -, no mutation detected by SSCP
mut. - mutation present by SSCP
loh - loss of heterozygosity noticed by SSCP
26: p53 exon 7: -, no mutation detected by SSCP
mut. - mutation present by SSCP
27: p53 exon 8: -, no mutation detected by SSCP
mut. - mutation present by SSCP
28: 17p loss as detected by CGH:
- not detected
+ present
29: Combined p53 status:
n - p53 functional
n*-p53 probably functional
d - p53 defective
? - undetermined

APPENDIX 2

ABBREVIATIONS

ABC	avidin/biotinylated horseradish peroxidase complex
ACF	aberrant crypt foci
APC	Adenomatous Polyposis Coli
ATM	ataxia telangiectasia mutated
BRCD2	breast cancer suppressor-2
cAMP	cyclic adenine monophosphate
CDC2L1	cell division cycle 2-like 1
CDK4	cyclin-dependent kinase 4
CEA	carcino-embryonic antigen
CGH	Comparative Genomic Hybridisation
CHRPE	congenital hypertrophy of the retinal pigment epithelium
CIN	chromosomal instability
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAN	differential-screening-selected gene aberrant in neuroblastoma
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
DCC	*d*eleted in *c*olon *c*ancer
DDW	distilled deionised water
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanine triphosphate
DLC1	*d*ynein *l*ight-*c*hain gene *1*
dNTP	ddeoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxynucleotide triphosphate
DPC4	*d*eleted in *p*ancreatic *c*ancer
dUTP	deoxyuracyl triphosphate
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EXTL3	exostoses (multiple)-like 3
FAP	Familial Adenomatous Polyposis
GTP	guanosine triphosphate
GTBP	G-T mismatch binding protein
H&E	haematoxilin and eosin
hMLH1	human MutH homologue
hMSH2	human MutS homologue 2
hMSH3	human MutS homologue 3
hMSH6	human MutS homologue 6
hPMS1	human post-meiotic segregation homologue 1
hPMS2	human post-meiotic segregation homologue 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HRP	horseradish peroxidase
ID3	inhibitor of DNA binding 3
IHC	immunohistochemistry
LOH	loss of heterozygosity
MAD	*m*others *a*gainst *d*pp
MCC	*m*utated in *c*olorectal *c*ancer
MDE	modified acrylamide (trade name)

MMR	mismatch repair
MIN	microsatellite instability
Min	*m*ultiple *i*nterstinal *n*eoplasia
Mom1	*m*odifier *o*f *m*in 1
mRNA	messenger ribonucleic acid
NB/NBS	neuroblastoma suppressor
NMSK	non-melanoma skin cancer
NRS	normal rabbit serum
OD	optical density
PLA2s	type II non-pancreatic phospholipase A2
PLPD	periodate-lysine-paraformaldehyde-dichromate
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PRLTS	*P*DGF-*r*eceptor β-*l*ike *t*umour *s*uppressor
QUIPS	Quantitative Image Processing System
RER	replication error
RER+	replication error positive
RER-	replication error negative
RNA	ribonucleic acid
SCID	severe combined immuno-deficient
SDS	sodium dodecyl sulphate
STR	short tandem repeat
SSC	salt/sodium citrate buffer
SSCP	single-stranded conformational polymorphism
TBE	tris-borate-EDTA
TE	tris-EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
TGFβ	transforming growth factor β
TGFβ RII	transforming growth factor β receptor subunit II
TNFR2	tumour necrosis factor receptor 2

APPENDIX 3

REAGENTS AND SUPPLIERS

Reagents are listed in alphabetical order against the name of their supplier(s).

96-well PCR plates; Hybaid Ltd
α^{32}PdCTP; Amersham International plc
ABC kit; Dako Ltd
Agarose; Sigma Aldrich Chemical Company
Alkaline phosphatase substrate kit BCIP/NBT; Vector Laboratories
Ammonium acetate; Fisher Scientific
Ammonium persulphate; Severn Biotech Ltd
Anti-digoxigenin rhodamine; Sigma Aldrich Chemical Company
BCIP/NBT alkaline phosphatase substrate kit; Dako Ltd
Bench alcohol; Genta Medical
Biotin-16 dUTP; Boehringer-Mannheim Ltd
Biotinylated anti-avidin; Vector Laboratories
Biotinylated rabbit anti-mouse immunoglobulins; Dako Ltd
Blocking agent; Amersham International plc
Boric acid; Sigma Aldrich Chemical Company
Bromophenol blue; Fisher Scientific
BSA fraction V; Fisher Scientific
Calcium chloride; Fisher Scientific
Chloroform; Fisher Scientific
Cot1 DNA, human and mouse; Life Technologies Ltd
Cover slips (No.0 & No.1, Chance Propper); Fisher Scientific
DEAE membrane; Schleicher and Schuell
'Decon' detergent; Fisher Scientific
DEPC; Sigma Aldrich Chemical Company
Diaminobenzidine (DAB); Sigma Aldrich Chemical Company
Diaminoethanetetra-acetic acid (EDTA), Sigma Aldrich Chemical Company
Diaminophenolindole (DAPI); Boehringer-Mannheim Ltd
Digoxygenin-11-dUTP; Boehringer-Mannheim Ltd
Dimethyl sulfoxide (DMSO); Sigma Aldrich Chemical Company
DNA mass ladder; Life Technologies Ltd
DNA molecular weight marker V; Boehringer-Mannheim Ltd
DNA molecular weight marker 1 kilobase DNA ladder; Life Technologies Ltd
DNA polymerase I; Life Technologies Ltd
DNase I; Boehringer-Mannheim Ltd
dNTPs; Pharmacia Biotechnologies Ltd
Do7 antibody; Dako Ltd
*Eco*RI + buffer; NBL Gene Science Ltd
Ethanol; Hayman Ltd
Ethidium bromide; Sigma Aldrich Chemical Company
Fluorescein-avidin DCS; Vector Laboratories
Formaldehyde solution; Fisher Scientific
Formamide: Fisher Scientific
γ^{33}PdATP; Amersham International plc
'Gel Slick'; AT Biochem
Glacial acetic acid; Fisher Scientific
Glycerol; Sigma Aldrich Chemical Company

Hybond N+; Amersham International plc
Hydrochloric acid; Fisher Scientific
Hydrogen peroxide; Sigma Aldrich Chemical Company
Ion-exchange resin beads; Bio-Rad Laboratories Ltd
Immuno-Check alignment fluorospheres; Coulter Electronics Ltd
'Instagel' 40% 19:1 acrylamide:bis acrylamide solution; Severn Biotech Ltd
Iso-amyl alcohol; Fisher Scientific
Kodak X-OMAT autoradiography film; Amersham International plc
L-glutamine; Life Technologies Ltd
Magnesium chloride; Sigma Aldrich Chemical Company
Magnesium Sulphate; Sigma Aldrich Chemical Company
MDE gel; AT Biochem
Methanol; Fisher Scientific
Microscope slides 'Select' (Chance Propper); Fisher Scientific
N,N,N',N',-tetramethylethylenediamine (TEMED); Severn Biotech Ltd
Nonidet P40; Fisher Scientific
Normal rabbit serum; Life Technologies Ltd
Phosphate buffered saline (PBS); Life Technologies Ltd
Phytohaemmagglutinin (M-form); Murex Diagnostics Ltd and Sigma Aldrich Chemical Company
Prime-It RmT kit; Stratagene
Potassium chloride; Fisher Scientific
Propidium iodide; Sigma Aldrich Chemical Company
Proteinase K; ICN Biomedicals Ltd
*Pst*I + buffer; NBL Gene Science Ltd
Quick Spin columns G50 (fine); Boehringer-Mannheim Ltd
RNase A; Sigma Aldrich Chemical Company
RPMI medium (Dutch modification); Life Technologies Ltd
Sephadex G-50 nick column; Pharmacia Biotechnologies Ltd
Sodium carbonate (decahydrate); Fisher Scientific
Sodium chloride; Fisher Scientific
Sodium citrate; Fisher Scientific
Sodium dodecyl sulphate (SDS); ICN Biomedicals Ltd
Sodium hydroxide; Fisher Scientific
Sodium dihydrogen orthophosphate; Fisher Scientific
Sodium thiosulphate; Fisher Scientific
Spermine tetrahydrochloride; Sigma Aldrich Chemical Company
Streptavidin alkaline phosphatase; Boehringer-Mannheim Ltd
Streptavidin alkaline phosphatase anti-digoxygenin; Boehringer-Mannheim Ltd
Sucrose; Fisher Scientific
T4 polynucleotide kinase + buffer; Life Technologies Ltd
Thermostable DNA polymerase + buffer IV; Advanced Biotechnologies Ltd
Tissue culture plasticware; Costar Ltd
Tris; Sigma Aldrich Chemical Company
Trisodium citrate; Sigma Aldrich Chemical Company
Trypsin; Sigma Aldrich Chemical Company
Trypsin Inhibitor; Sigma Aldrich Chemical Company
Tween 20; Fisher Scientific
Urea; Fisher Scientific
'Vectashield' mounting medium; Vector Laboratories
Water-saturated phenol; Rathburn Chemicals
Whatman paper; Whatman Ltd
Xylene; Fisher Scientific
Xylene cyanol FF; Sigma Aldrich Chemical Company

APPENDIX 4

SUPPLIERS' ADDRESSES

Advanced Biotechnologies Ltd
Units B1-B2
Longmead Business Centre
Blenheim Road
Epsom
Surrey
KT19 9QQ

Amersham International plc
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA

AT Biochem
30 Spring Mill Drive
Malvern
PA 19355
USA

Boehringer-Mannheim UK (Diagnostics and Biochemicals) Ltd
Bell Lane
Lewes
E. Sussex
BN7 1LG

Bio-Rad Laboratories Ltd
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Hertfordshire
HP2 7TD

Costar UK Ltd
10 The Valley Centre
Garden Road
High Wycombe
Buckinghamshire
HP13 6EQ

Coulter Electronics Ltd
Luton
Bedfordshire

Dako Ltd
16 Manor Courtyard
Hughenden Avenue
High Wycombe
Buckinghamshire
HP13 5RE

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire
LE11 ORG

Genta Medical
Marston Business Park
Rudgate
Tockwith
York
YO5 8QF

Hayman Ltd
70 Eastways Industrial Park
Witham
Essex
CM8 3YE

Hybaid Ltd
111-113 Waldegrave Road
Teddington
Middlesex
TW11 8LL

ICN Biomedicals Ltd
Unit 18
Thame Park Business Centre
Wenman Road
Thame
Oxfordshire
OX9 3XA

Life Technologies Ltd
3 Fountain Drive
Inchinnin Business Park
Paisley
PA4 9RF

NBL Gene Science Ltd
South Nelson Industrial Estate
Cramlington
Northumberland
NE23 9HL

Pharmacia Biotechnologies Ltd
Davy Avenue
PO Box 100
Knowlhill
Milton Keynes
MK5 8PB

Rathburn Chemicals Ltd
Walkerburn Scotland
EH46 6AU

Severn Biotech Ltd
Unit 2
Park Lane
Kidderminster
Worcestershire
DY11 6TJ

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH17 7NH

Stratagene Ltd
Cambridge Innovation Centre
140 Cambridge Science Park
Milton Road
Cambridge
CB4 4GF

Vector Laboratories
16 Wulfric Square
Bretton
Peterborough
PE3 8RF

Vysis (UK) Ltd
Rosedale House
Rosedale Road
Richmond
Surrey
TW9 25Z

Whatman International Ltd
St Leonard's Road
20/20 Maidstone
Kent
ME16 OLS

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS.

Publications:

WM Abdel-Rahman, IB Georgiades, LJ Curtis, MJ Arends and AH Wyllie.
"Role of BAX mutation in mismatch repair-deficient colorectal carcinogenesis" *Oncogene*, 1999, Vol.18, No.12, pp 2139-2142.

IB Georgiades, LJ Curtis, RG Morris, CC Bird and AH Wyllie.
"Heterogeneity studies identify a subset of sporadic colorectal cancers without evidence for chromosomal or microsatellite instability" *Oncogene*, 1999, Vol.18, No.56, pp 7933-40.

LJ Curtis, IB Georgiades, S White, CC Bird and AH Wyllie.
"Specific patterns of chromosomal abnormalities are associated with RER status in sporadic colorectal cancer" *Journal of Pathology*, 2000, Vol.192, pp 440-445.

Presentations:

IB Georgiades, LJ Curtis, RG Morris, AH Wyllie.
"Heterogeneity studies identify a substantial subset of sporadic colorectal cancers without chromosomal or microsatellite instability" - presented at the poster presentation at Keystone Symposium on The Molecular Basis of Cancer in Taos, New Mexico,17 March 1999.

IB Georgiades, LJ Curtis, AH Wyllie.
"Intratumoral heterogeneity in colorectal cancer and the clonal evolution of imbalanced chromosomal changes" - presented at the poster presentation at the Summer Meeting of the Pathological Society of Great Britain and Ireland in Leicester, 2 July 1998.

Oncogene (1999) 18, 2139–2142

http://www.stockton-press.co.uk/onc

Role of *BAX* mutations in mismatch repair-deficient colorectal carcinogenesis

Wael M Abdel-Rahman[1], Izabela B Georgiades[1], Lucy J Curtis[1], Mark J Arends[1] and Andrew H Wyllie*,[1]

[1]*Sir Alastair Currie CRC Laboratories, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU*

***BAX* gene mutations occur in approximately 50% of RER+ colorectal cancers. To determine the role of these mutations in tumour progression we analysed multiple different tumour sites from RER+ colorectal cancers for *BAX* mutations. Sixty colorectal carcinomas were analysed for microsatellite instability at loci BAT-26, L-myc, TGFβRII, D13S160 and D2S123. Twelve out of 60 tumours (20%) were RER+. Forty-five different tumour sites from the 12 RER+ carcinomas were analysed for *BAX* mutations at the [(G)8] tract in exon 3. Six out of 12 (50%) RER+ tumours showed *BAX* mutations, four of which showed a homogenous pattern of such mutations detected in all tumour sites. In the other two cases, *BAX* mutations were present in some but not all tumour sites sampled from the same patient. In contrast, *TGFβRII* mutations were found in 9/12 cases (75%) and in each of these were present in all the sampled sites. Two cases showed neither *BAX* nor *TGFβRII* mutation. These data suggest that mutations in *TGFβRII* may occur at a very early stage in tumour progression, perhaps in the founder clone. *BAX* mutations, however, are clearly not necessary for formation of the founder clone and can occur for the first time later in tumour progression.**

Keywords: *BAX*; colorectal cancer; RER; mismatch repair

Introduction

The BCL2 family of proteins control an important checkpoint prior to activation of the caspase family of proteases in apoptosis (Brown, 1997; White, 1996). One prominent, widely expressed member of this family is BAX, a 21 kDa protein with the capacity to homodimerize or heterodimerize with other members of the BCL2 family (Oltvai *et al.*, 1993). Whereas BAX-BAX homodimers are potent death inducers, the BAX-BCL2 heterodimers and BCL2-BCL2 homodimers appear to promote survival (Kroemer, 1997). Moreover, the tumour suppressor protein p53, a major element in the response to lethal stimuli arising from DNA damage or hypoxia, can transactivate *BAX* (Miyashita and Reed, 1995) whilst down-regulating *BCL2* (Miyashita *et al.*, 1994). BAX is thus a significant effector in the initiation of apoptosis.

*Correspondence: AH Wyllie, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP
Received 3 August 1998; revised 11 November 1998; accepted 24 November 1998

Recently, clonally expanded, inactivating mutations in *BAX* have been observed in a proportion of colorectal cancers, together with evidence for under-expression of the BAX protein in these tumours (Rampino *et al.*, 1997; Yamamoto *et al.*, 1998; Yagi *et al.*, 1998; Ouyang *et al.*, 1998). This provides some circumstantial evidence for the hypothesis that the founder cells of cancers may arise through selective loss of a death pathway and the resultant inappropriate survival of cells that have sustained DNA damage or other severe intracellular injury. This hypothesis carries the significant implication that cancer cells that arise in this way are liable to be resistant to many cytotoxic agents to which more normal cells would be sensitive, because of constitutional loss of a critical death pathway. Alternative explanations for the *BAX* mutations exist, however. These mutations are restricted to tumours with mismatch repair (MMR) deficiency, and usually occur in a tract of eight consecutive deoxyguanosines [(G)8] in the third coding exon (Rampino *et al.*, 1997). Since this is a classical target site for nucleotide mismatch (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993), it is possible that mutations within [(G)8] simply reflect the well-recognized effect of MMR deficiency on mutation incidence in tandem repeat microsatellite sequences.

In this paper we seek to distinguish between these possibilities by studying the homogeneity of *BAX* mutation within colorectal cancers. We argued that genetic changes that are critical for carcinogenesis are likely to be shared by all cells in the expanding tumour and should therefore be detectable at all sites throughout the tumour. In contrast, changes that reflect the genomic instability of malignant cells, but are not essential for the transition to malignancy might be expected to occur in some but not all of the divergent subclones within the given tumour. Accordingly, in this paper we studied *BAX* mutations in the [(G)8] tract in a series of tumours sampled at multiple sites.

Results

RER characterization

Twelve out of 60 patients (20%) demonstrated microsatellite instability (Table 1). In some cases (nos. 17, 27, 52, 53 and 55) individual tumour sites from the same carcinoma demonstrated different sets of mutations at the five microsatellite loci tested. In some tumours, biopsies from different sites each exhibited different mutations at the same microsatellite locus

Table 1 Mismatch repair deficient carcinomas analysed for RER and *BAX* status

No	*Age*	*Sex*	*Site*	*Histology*	*Sample site*	*RER status* *BAT-26*	*L-Myc*	*TGFβRII*	*D13S160*	*D2S123*	Bax *mutation*
17	81	F	cae	mucoid	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	–	+	+	–	+	–
18	67	F	sig	ac md	A	+	–	–	–	–	–
					B	+	–	–	–	–	–
					C	+	–	–	–	–	–
19	70	M	asc	ac pd	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	+	+	+	–	+	+(loss)
22	43	F	cae	ac/mu	A	+	–	–	–	–	+(loss)
					B	+	–	–	–	–	+(loss)
25	65	F	cae	ac/mu	A	+	+	+	+	–	+(loss)
					B	+	+	+	+	–	+(loss)
					C	+	+	+	+	–	–
					D	+	+	+	+	–	+(loss)
27	88	F	asc	ac/mu	A	+	+	+	–	+	–
					B	+	+	+	+	+	–
					C	+	+	+	+	+	–
					D	+	+	+	+	+	–
					E	+	+	+	+	+	–
					F	+	+	+	+	+	–
					G	–	+	+	–	+	–
28	75	F	cae	ac/mu	A	+	+	+	+	–	–
					B	+	+	+	+	–	–
38	64	M	asc	ac md	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	+	+	+	–	+	+(loss)
					D	+	+	+	–	+	+(loss)
47	60	M	asc	ac pd	A	+	–	+	+	+	–
					B	+	–	+	+	+	–
					C	+	–	+	+	+	–
					D	+	–	+	+	+	–
52	76	M	cae	ac pd	A	+	+	+	+	–	–
					B	+	+	+	+	–	–
					C	+	+	+	+	+	–
					D	+	+	+	+	+	–
53	77	F	asc	ac pd	A	+	+	–	–	–	–
					B	+	+	–	+	–	–
					C	+	+	–	–	+	–
					D	+	+	–	–	–	–
55	70	F	asc	ac pd	A	+	+	+	+	–	+(gain)
					B	+	+	+	–	–	+(gain)
					C	+	+	+	+	–	+(gain)
					D	+	+	+	–	–	+(gain)

Cae = caecum, asc = ascending colon, sig = sigmoid colon, mucoid = mucoid carcinoma, ac md = moderately differentiated adenocarcinoma, ac pd = poorly differentiated adenocarcinoma, ac/mu = mixed adenocarcinoma glandular/mucoid pattern, +(loss) = deletion of one G from the [(G)8] tract, +(gain) = insertion of one G in the [(G)8] tract

(Figure 1). However, in no case was microsatellite instability present at one tumour site, and completely absent at all tested loci in others. Hence it was possible to classify all sampled sites of all tumours as RER+, although in two tumours (nos. 18 and 22) the instability was evident in only one of the five tested loci.

BAX *mutation analysis*

Mutation in the *BAX* [(G)8] mononucleotide repeat tract occurred in six out of 12 (50%) RER+ tumours. In five, this involved loss of a repeated nucleotide and in one a gain. Four showed a homogenous pattern, with identical changes in [(G)8] detected in all sites sampled from each carcinoma (Figure 2a and Table 1). In the other two cases, however, [(G)8] *BAX* mutations were present in some but absent in other sites from the same cancers. Thus, case number 17 showed mutation in tumour sites A and B, but not C and case number 25 showed mutations in A, B and D but not C (Figure 2b and Table 1). In both these cases, the tumour site in which *BAX* [(G)8] was unchanged showed unequivocal evidence of instability at most of the other tested microsatellite loci.

Discussion

It is clear that deficiency in mismatch repair permits the generation of large numbers of mutations throughout the genome, mainly in microsatellite loci and repetitive polynucleotide tracts (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993). In sporadic colorectal tumours, the microsatellite instability is generally found in carcinomas but not adenomas, whereas both adenomas and carcinomas from HNPCC patients may show a high proportion of such instability (Samowitz and Slattery, 1997; Bubb *et al.*, 1996; Jacoby *et al.*, 1995; Thibodeau *et al.*, 1993). These observations strongly suggest that microsatellite instability is acquired at the adenoma-carcinoma interface in the evolution of sporadic tumours, but can appear at an

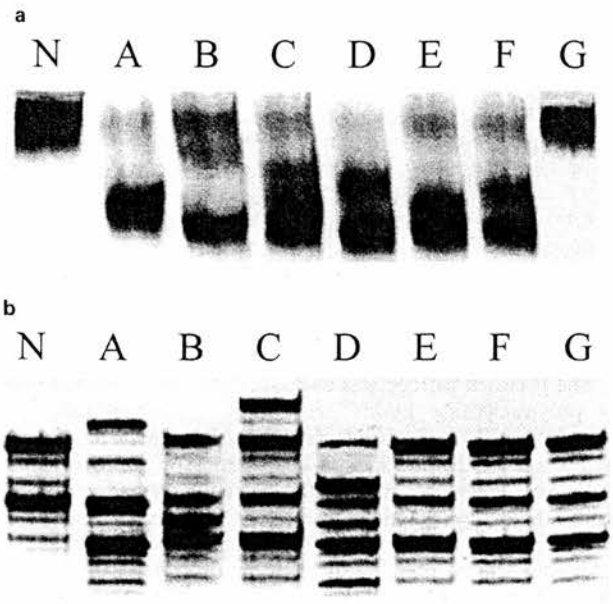


Figure 1 The band shift pattern of case no. 27 at the BAT-26 (**a**) and L-myc (**b**) microsatellite loci (N = normal mucosa; A-G = 7 different tumour sites from the same carcinoma)

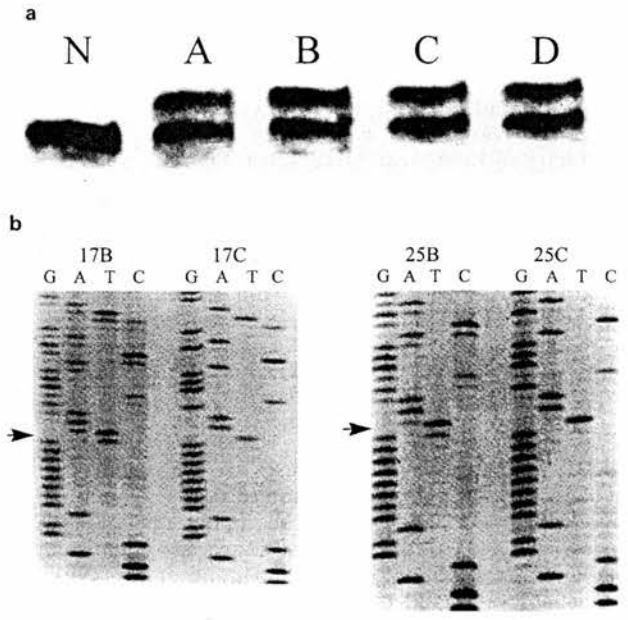


Figure 2 (**a**) *BAX* gene analysis of case no. 55 showing the band shift pattern on a polyacrylamide gel demonstrating one nucleotide insertion in the [(G)8] tract from all tumour sites (A–D), compared to normal mucosa (N). (**b**) Sequence analysis of case 17 (B and C) and 25 (B and C). In both cases, site C does not show the one nucleotide deletion in the [(G)8] tract observed in site B (indicated by arrow)

earlier stage in patients who carry germline mutations in MMR genes. Previous studies have shown clonal expansion of shifts of different amplitude at the same microsatellite locus sampled from different sites in the same tumour (Chung *et al.*, 1997), as observed in the present work also. This indicates that a proportion of these microsatellite mutations are acquired as clonal variants throughout the process of tumour formation and reflect but do not cause the evolution of such tumours. In contrast, mutations at some genetic loci have been found with great consistency in RER+ tumours. An outstanding example is transforming growth factor beta-type 2 receptor (TGFβRII), which is mutated in upwards of 90% of all tested RER+ colorectal cancers (Markowitz *et al.*, 1995; Parsons *et al.*, 1995). In this series, more than 75% of RER+ cancers showed a mutation in the one site within the TGFβRII gene which we tested. Together with independent evidence that TGFβ exerts a suppressive effect on colorectal epithelial growth (Wrana *et al.*, 1994), these observations have been interpreted as indicative of a causal role for TGFβRII inactivation in colorectal carcinogenesis.

Frameshift mutations have been detected in the [(G)8] tract of exon 3 of the *BAX* gene in 48% of 63 RER+ sporadic colorectal cancers in a total of two separate studies (Rampino *et al.*, 1997; Ouyang *et al.*, 1998), and in a similar proportion of RER+ colorectal cancers from HNPCC patients (Yamamoto *et al.*, 1998; Yagi *et al.*, 1998). Our own observations are entirely concordant with these results: we detected *BAX* mutations in six out of 12 (50%) RER+ carcinomas. In the presence of *BAX* and TGFβRII mutations, others have shown second allele mutations in pure cultures of cell lines (Rampino *et al.*, 1997; Markowitz *et al.*, 1995), but this is much more difficult to demonstrate in primary tumours due to contamination by stromal and lymphoid cells. Hence some uncertainty remains over the functional status of BAX in primary human tumours, even when there is evidence for mutation in one allele. However, we report here, we believe for the first time, two patterns for such mutations. In four of the six tumours bearing *BAX* mutations, identical alterations in the [(G)8] tract were found in all sites sampled within each cancer, supporting the hypothesis that *BAX* mutation was present in the founder malignant clone. This pattern is also consistent with that described for *BAX* mutations in gastric cancers (Chung *et al.*, 1997). However, in two of six cases we demonstrated a second pattern in which *BAX* mutation is not shared by all the tumour sites of the same cancer. We can not completely exclude the possibility that the apparently unaltered [(G)8] tract found in subclones of these tumours represents a reversion, through a second mutation, of the [(G)8] mutation present elsewhere. This possibility can in the future be tested in appropriate cell culture models. A more obvious explanation, however, is that in these tumours the *BAX* [(G)8] mutation was not present in the founder malignant clone but was acquired later in cancer progression. In these tumours, it is difficult to sustain the view that mutational inactivation of *BAX* could have been a critical event early in carcinogenesis. These data therefore raise some doubt as to the significance of loss of BAX-dependent apoptosis pathways in colorectal carcinogenesis. Rather than indicating that failure of apoptosis exerts a critical role in carcinogenesis, some *BAX* mutations in colorectal tumours may merely aid tumour progression, or indeed may simply reflect the consequences of mismatch repair deficiency without a functional connotation.

Materials and methods

Tissue samples

Fresh tumour samples were collected from patients with colorectal carcinoma undergoing surgery in the Royal Infirmary of Edinburgh NHS Trust. Samples were collected from the operating theatre within 30 min of resection and transported to the Pathology Department. Two to seven small tumour pieces and the matched normal tissues from each case were frozen in liquid nitrogen, then stored at −70°C. DNA was extracted from frozen tissues by the method of Goelz *et al.* (1985).

RER status analysis

Sixty tumours were originally screened for microsatellite instability and only RER+ cases were analysed for *BAX* gene mutation. All the samples were tested at five microsatellite loci including the highly unstable BAT-26 locus, claimed to be sufficient alone for identifying the RER status (Zhou *et al.*, 1998; Hoang *et al.*, 1997). The other 4 loci included L-myc, TGFβRII, D13S160, and D2S123 using the primers and conditions described elsewhere (Young *et al.*, 1995; Huang *et al.*, 1996).

BAX *gene analysis*

Twelve RER+ cases with a total of 45 different tumour sites and the matched normal tissue constituted in the material for *BAX* gene mutational analysis.

A DNA segment of 94-base pairs encompassing the [(G)8] tract in *BAX* was amplified by PCR using the primers described by Rampino *et al.* (1997). PCR was carried out for 30 cycles, each consisting of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C. Reactions consisted of 50 ul volume containing 100 ng genomic DNA, 10 pmols of each primer, 200 uM of each dNTP (Advanced Biotechnologies Ltd), 1.5 mM $MgCl_2$, 1.25 U of thermostable Taq DNA polymerase and buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 0.05% non-ionic detergent (Life Technologies UK). The PCR hot start method was used. The products were analysed by electrophoresis on 2% agarose gels for detection of the amplified product.

The forward primer was end labelled with γ-^{33}P-ATP using T4 polynucleotide kinase according to the manufacturer's instructions (Life Technologies UK). This primer was then used to label the original PCR product by performing one PCR cycle using the same conditions as described above. The radio-labelled products were electrophoretically separated in denaturing 6% polyacrylamide gel and subjected to autoradiography for detection of band shifts. At the same time, all the original PCR products were directly sequenced with Thermosequenase radiolabelled terminator cycle sequencing kits (Amersham Life Science).

Acknowledgements
The authors' work was supported by grants from Cancer Research Campaign, Association for International Cancer Research, Scottish Hospitals Endowment Research Trust, and Egyptian Education Bureau-London.

References

Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B and Delachapelle A. (1993). *Science*, **260**, 812–816.

Brown R. (1997). *Br. Med. Bull.*, **53**, 466–477.

Bubb VJ, Curtis LJ, Cunningham C, Dunlop MG, Carothers AD, Morris RG, White S, Bird CC and Wyllie AH. (1996). *Oncogene*, **12**, 2641–2649.

Chung YJ, Park SW, Song JM, Lee KY, Seo EJ, Choi SW and Rhyu MG. (1997). *Oncogene*, **15**, 1719–1726.

Goelz SE, Hamilton SR and Vogelstein B. (1985). *Biochem. Biophys. Res. Commun.*, **130**, 118–126.

Hoang JM, Cottu PH, Thuille B, Salmon RJ, Thomas G and Hamelin R. (1997). *Cancer Res.*, **57**, 300–303.

Huang J, Papadopoulos N, Mckinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JKV, Markowitz SD, Morin P, Kinzler KW, Vogelstein B and Dunlop MG. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 9049–9054.

Jacoby RF, Marshall DJ, Kailas S, Schlack S, Harms B and Love R. (1995). *Gastroenterology*, **109**, 73–82.

Kroemer G. (1997). *Nature Med.*, **3**, 614–620.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun LZ, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M and Willson JKV. (1995). *Science*, **268**, 1336–1338.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene*, **9**, 1799–1805.

Miyashita T and Reed JC. (1995). *Cell*, **80**, 293–299.

Oltvai ZN, Milliman CL and Korsmeyer SJ. (1993). *Cell*, **74**, 609–619.

Ouyang H, Furukawa T, Abe T, Kato Y and Horii A. (1998). *Clin. Cancer Res.*, **4**, 1071–1074.

Parsons R, Myeroff LL, Liu B, Willson JKV, Markowitz SD, Kinzler KW and Vogelstein B. (1995). *Cancer Res.*, **55**, 5548–5550.

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. (1997). *Science*, **275**, 967–969.

Samowitz WS and Slattery ML. (1997). *Am. J. Pathol.*, **151**, 33–35.

Thibodeau SN, Donovan KA, Tester DJ, Bren GD, Halling KC, Cunningham J, Morris AV and Roche PC. (1993). *Am. J. Hum. Genet.*, (Suppl.), **53**, 1.

White E. (1996). *Genes Dev.*, **10**, 1–15.

Wrana JL, Attisano L, Wieser R, Ventura F and Massague J. (1994). *Nature*, **370**, 341–347.

Yagi OK, Akiyama Y, Nomizu T, Iwama T, Endo M and Yuasa Y. (1998). *Gastroenterology*, **114**, 268–274.

Yamamoto H, Sawai H, Weber TK, RodriguezBigas MA and Perucho M. (1998). *Cancer Res.*, **58**, 997–1003.

Young J, Searle J, Buttenshaw R, Thomas L, Ward M, Chenevixtrench G and Leggett B. (1995). *Genes Chrom. Cancer*, **12**, 251–254.

Zhou XP, Hoang JM, Li YJ, Seruca R, Carneiro F, SobrinhoSimoes M, Lothe RA, Gleeson CM, Russell SEH, Muzeau F, Flejou JF, HoangXuan K, Lidereau R, Thomas G and Hamelin R. (1998). *Genes Chrom. Cancer*, **21**, 101–107.

Oncogene (1999) 18, 7933–7940

http://www.stockton-press.co.uk/onc

Heterogeneity studies identify a subset of sporadic colorectal cancers without evidence for chromosomal or microsatellite instability

IB Georgiades[1,3], LJ Curtis*[,1], RM Morris[1], CC Bird[1,2] and AH Wyllie[1,4]

[1]*Sir Alastair Currie CRC Laboratories, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK;* [2]*Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK*

Two apparently independent mechanisms of instability are recognized in colorectal cancer, microsatellite instability and chromosomal instability. Evidence from colorectal cancer cell lines indicates the presence of either, or both, types of instability in the vast majority. Here, we sought to determine the prevalence of such instability in primary sporadic colorectal cancers. Microsatellite instability was established by demonstration of ovel clonal, nongerm-line alleles in at least two of four tested loci. Chromosomal abnormalities were identified by comparative genomic hybridization (CGH) and flow cytometric analysis of nuclear DNA content. Tumours harbouring chromosomal instability were distinguished from those with stable but aneuploid karyotypes by comparing chromosomal defects at multiple sites throughout each cancer. This analysis allowed assessment of both the number of chromosomal abnormalities and their heterogeneity throughout the tumour. The results confirm that microsatellite instability is consistently associated with multiple, repeated changes in microsatellites throughout the growth of the affected colorectal carcinomas. There were also several carcinomas in which major structural or numerical abnormalities in chromosomes had clearly continued to arise during tumour growth. However, a substantial subset of tumours showed neither microsatellite instability nor multiple, major chromosomal abnormalities. We suggest that the development of a proportion of colorectal cancers proceeds via a different pathway of carcinogenesis not associated with either of the currently recognized forms of genomic instability.

Keywords: colorectal cancer; chromosomal instability; genomic instability; microsatellite instability; RER

Introduction

The development of genetic instability has been proposed as an important event in multi-step carcinogenesis (Loeb, 1991; Hartwell, 1992). In human colorectal carcinoma (probably the human tumour most intensively studied at the genetic level) two major mechanisms of genomic instability have been identified. The first, known as microsatellite instability (MIN), manifests as a high rate of alteration in the length of short tandemly repeated nucleotide sequences (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Eshleman *et al.*, 1995). Such instability is a characteristic of tumours from patients with Hereditary Non-Polyposis Colorectal Cancer (HNPCC) where it is consequent upon germ-line mutations in DNA mismatch repair (MMR) genes (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3* or *hMSH6*) (Peltomaki *et al.*, 1993; Bronner *et al.*, 1994; Nicolaides *et al.*, 1994; Nystrom-Lahti *et al.*, 1994; Papadopoulos *et al.*, 1994; Wijnen *et al.*, 1995; Liu *et al.*, 1996; Akiyama *et al.*, 1997; Miyaki *et al.*, 1997). Defects in these genes result in multiple inaccuracies in replication of such tandem arrays, creating frameshifts and, occasionally, point mutations: the RER+ phenotype. Many genes include potential target sequences for this type of error, and mutation in some of these may confer growth advantage, and so be selected for during tumorigenesis (Markowitz *et al.*, 1995; Eshleman *et al.*, 1996; Togo *et al.*, 1996; Rampino *et al.*, 1997). The RER+ phenotype is also detected in 15–20% of sporadic colorectal cancers (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Wu *et al.*, 1994; Borresen *et al.*, 1995; Bubb *et al.*, 1996; Eshleman and Markowitz, 1996; Konishi *et al.*, 1996; Liu *et al.*, 1996). Here, its origins are conjectural, although acquired bi-allelic mutation in mismatch repair genes is responsible for some, and suppression of their activity by other mechanisms appears to occur in the majority of the remainder (Thibodeau *et al.*, 1998).

The majority of sporadic colorectal cancers, however, do not show the RER+ phenotype. RER− colorectal cancers, unlike RER+ tumours, frequently show major abnormalities in chromosome structure and number, and it has been suggested that these tumours arise through chromosomal instability (CIN). Loss of a mitotic checkpoint may account for repeated errors in chromosome disjunction in many of these tumours, and loss of function of *hBUB1*, a critical mitotic checkpoint gene, has been observed in a small proportion of colorectal cancer cell lines exhibiting chromosomal instability (Cahill *et al.*, 1998). However, other mechanisms must exist, permitting the growth of clones of cells that have sustained chromosome breakage, fusion, deletion and amplification events. Telomere erosion, hypomethylation and dysfunction of p53 (all phenomena that are observed frequently and at an early stage in carcinoma development) may all be permissive for these events.

In this paper we demonstrate substantial heterogeneity amongst RER− colorectal cancers. To avoid distortion due to selection for growth potential *in vitro*,

*Correspondence: LJ Curtis
Current addresses: [3]Department of Histopathology, The General Infirmary at Leeds, Great George Street, Leeds, LS1 3CX, UK; [4]Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK
Received 20 July 1999; revised 30 September 1999; accepted 22 November 1999

we based our study on primary colorectal tumours, removed at potentially curative operations from otherwise unselected patients. In classifying these tumours we devised means of estimating the extent of chromosome change based upon multiple sampling (to measure clonal divergence) and, in some instances, sampling of xenografts to detect sequential changes in real time. We identify around one third of RER− cancers in which the extent and frequency of chromosome change is much less than the majority, and close to that observed in many RER+ tumours.

Results

Numerical measurement of chromosome instability

To quantify and compare the level of chromosomal instability in both groups of colorectal cancer we first carried our comparative genomic hybridization (CGH) analysis on samples collected from multiple sites from each tumour. The mean number of chromosomal changes would be expected to be high in tumours with underlying chromosomal instability and might vary between different sites sampled from the same tumour due to clonal divergence. However, the presence of chromosomal changes might equally represent a single catastrophic genomic event, resulting in an aneuploid but stable genome subsequently carried by all or the majority of tumour cells. In such cases, chromosomal changes would be expected to be similar in all or the majority of the sites examined. To distinguish these events we scored separately the sum of the mean number of chromosomal arm gains and losses detected in each tumour and a heterogeneity score, which was the number of particular chromosomal changes which were not consistent between different sites within a tumour (for example see Figure 1). Added together, these two scores constituted a 'chromosomal instability index'. A high heterogeneity score indicates an ongoing process of chromosomal gain and loss, despite the fact that some of the changes might have been selected for early during tumour progression. In practice, the majority of tumours showed heterogeneity scores roughly proportional to their mean numbers of chromosomal gains and losses. In such tumours, either score would provide a measure of 'chromosome instability' (Figure 2). In a small number of tumours, however, despite sampling at a similar number of sites to the majority, high numbers of gains and losses were not accompanied by pronounced intratumoral heterogeneity, showing that these two parameters could in some circumstances be independent.

Chromosomal instability in RER+ and RER− tumours

The mean number of chromosomal changes, heterogeneity scores and the combined CIN index scores in relation to the tumours' RER status are shown in Table 1. The mean number of chromosomal gains and losses and the CIN index were both significantly higher in RER− colorectal cancers compared with RER+ tumours (one-tailed Mann-Whitney Test, $P = <0.05$ for both comparisons). Scores for RER− tumours were scattered over a wide range, however, and we identified a substantial subgroup with low CIN indices (Figure 3). A tumour's CIN index was deemed to be low if it fell below an arbitrary value of 18 (which was the mean CIN index in RER+ cancers, a group generally believed to have little chromosomal instability).

CGH measures only relative abundances of DNA and would not identify as abnormal perfect tetraploid or octapoid genomes. We therefore carried out flow cytometric analysis of the nuclear DNA content of all tumour samples (Table 2a,b). As expected, the cells of most of the RER+ tumours showed near-diploid DNA content although, as recorded by ourselves and others elsewhere, abnormalities detectable by CGH were frequently present. The proportion of tumours with aneuploid content was significantly higher in the RER− group of tumours compared to RER+ cancers (two-tailed Fisher exact test, $P = <0.05$). Most RER− colorectal cancers with a low CIN index (four out of six) and all RER+ tumours with CIN index <18 showed diploid DNA content.

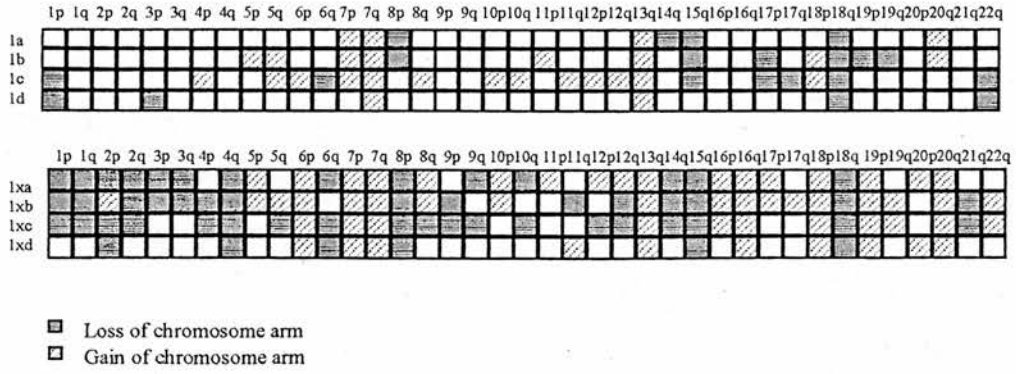


Figure 1 Example calculation of heterogeneity score and CIN index. CGH results in a primary tumour (tumour no. 1) sampled at four different sites, and in its four corresponding xenografts. Chromosome arms are represented vertically in columns, whilst each different site of the same tumour designated a, b, c and d are in rows. 1xa, 1xb, 1xc, and 1xd are the corresponding xenografts established from sites a, b, c and d. The heterogeneity score was calculated for the primary tumour and, separately, for the xenograft by adding together the number of columns representing chromosome arms in which changes were inconsistent between different sites within the tumour. In this primary tumour, inconsistent chromosome changes were present in chromosome arms 1p, 3p, 4p, 5p, 5q, 6p, 6q, 7p, 8p, 8q, 10p, 10q, 11p, 11q, 12p, 12q, 14q, 15q, 17p, 17q, 18p, 19p, 19q, 20q and 22q, giving a total heterogeneity score of 25. Consistent patterns, such as are present in 1q or 7q, do not score. CIN index was then calculated by adding together the mean number of chromosomal gains and losses and the heterogeneity score

p53 status and chromosomal instability in RER− and RER+ colorectal cancer

To investigate the role of p53 in chromosomal instability, we classified tumours as showing evidence of abnormality in p53 by three criteria; stabilization of the p53 protein as detected by immunohistochemistry, mutation analysis of exons 5–8 of the *p53* gene (screened by single stranded conformational polymorphism analysis, SSCP) and loss of the short arm of chromosome 17. The proportion of RER− and RER+ tumours with a p53 defect (mutation or immunohistochemical stabilization) was not significantly different (10/17 and 2/5, respectively) (Table 2a,b). The proportion of p53-defective tumours was significantly higher (10/13) among tumours with high CIN (above 18) compared to tumours with a low CIN index (2/9), regardless of RER status (two-tailed Fisher exact test, $P<0.05$). Tumours in which CGH revealed 17p loss, but which showed no positive IHC staining or presence of a mutation in exons 5–8 of the *p53* gene are indicated in Table 2 as n^*, but for the purpose of statistical analysis were treated as not defective for p53.

Clinicopathological features and chromosomal abnormalities in RER− tumours with low levels of chromosomal instability

Of the 22 primary colorectal carcinomas studied, eight were right-sided and 14 left-sided (Table 2a,b). Clinicopathological features of RER+ sporadic colorectal cancers have been described in detail (Lothe *et al.*, 1993; Aaltonen *et al.*, 1994; Kim *et al.*, 1994; Senba *et al.*, 1998) and our data are in concordance with these results (Table 2b): of the five studied, all were right-sided, four were near-diploid in DNA content, four were poorly differentiated and one showed abundant mucinous differentiation. The 6 RER− cancers with low CIN index were uniformly left-sided (compared with eight of 11 high-CIN RER− tumours [Table 2a]) and mostly rectal. There was no significant difference between the low CIN index and high CIN index RER− cancers with regard to Dukes' stage or patient age. Four RER− cancers with low CIN index were moderately differentiated adenocarcinomas, but a poorly differentiated tumour was also observed.

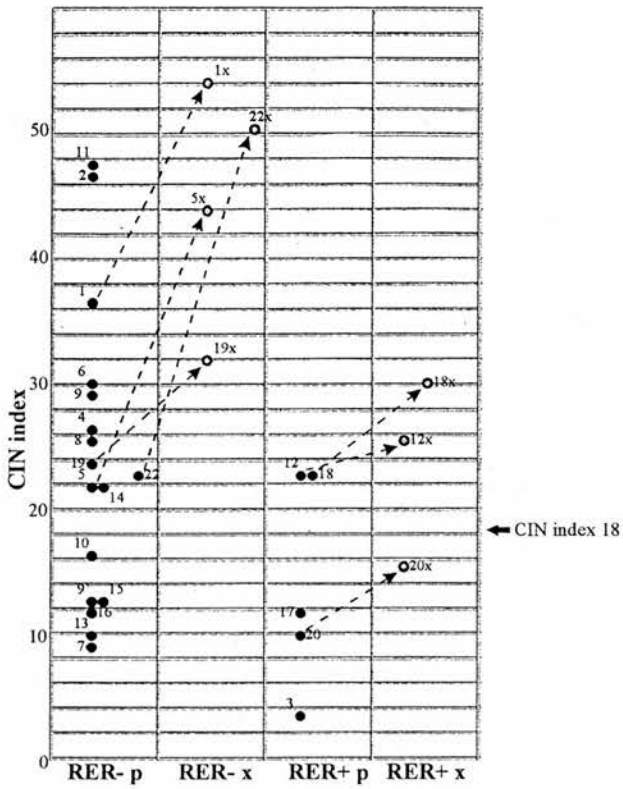


Figure 3 CIN index in RER− and RER+ primary colorectal cancers and corresponding xenograft. RER− p, RER− primary tumours (black points); RER− x, RER− xenografts (grey points); RER+ p, RER+ primary tumours (black points); RER+ x, RER+ xenografts (grey points). Numbers shown against each point indicate sample ID, listed in Table 2

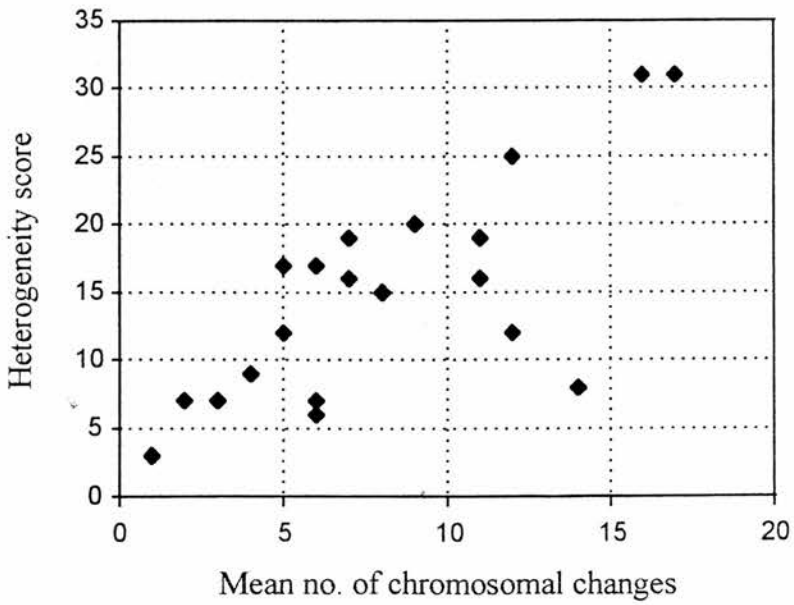


Figure 2 Mean number of chromosomal changes plotted against heterogeneity score in primary sporadic colorectal cancers. Two pairs of tumours had identical score for both values (see Table 1).

Table 1 Chromosomal instability in RER− and RER+ primary colorectal cancers and their corresponding xenografts

	Primary tumors			*Xenografts (if available from mutilple sites)*				
Sample	*Mean number of chromosomal changes*	*Heterogeneity score*	*CIN index*	*Sample*	*Mean number of chromosomal changes*	*Heterogeneity score*	*CIN index*	*Average xenograft age (weeks)*
RER−								
1	12	25	37	1x	27	27	54	15
2	16	31	47	na				
4	11	16	27	na				
5	5	17	22	5x	17	27	44	12
6	11	19	30	na				
7	2	7	9	na				
8	7	19	26	na				
9	9	20	29	na				
9′	4	9	13	na				
10	5	12	17	na				
11	17	31	48	na				
13	3	7	10	na				
14	14	8	22	na				
15	6	7	13	na				
16	6	6	12	na				
19	12	12	24	19x	25	7	32	12
22	8	15	23	22x	26	24	50	9.5
RER+								
3	1	3	4	na				
12	7	16	23	12x	10	16	26	4.5
17	6	6	12	na				
18	6	17	23	18x	13	17	30	12.5
20	3	7	10	20x	9	7	16	11

na, not available

Table 2

Sample	*Patient's age*	*DNA content*	*p53 status*	*Duke's stage*	*Tumour site/side*	*Differentiation*	*Mucinous*	*Xenograft established (any site)*
(a) p53 status, DNA content and clinicopathological data for RER− colorectal cancers								
CIN index > 18								
1	55	aneuploid	n*	C	asc.col./right	moderate	no	yes
2	62	aneuploid	d	B	rectum/left	moderate	no	no
4	72	aneuploid	d	B	rectum/left	moderate	no	yes
5	81	aneuploid	n*	B	sigm.col./left	moderate	no	yes
6	63	aneuploid	d	A	sigm.col./left	good	no	no
8	80	aneuploid	d	B	rectum/left	moderate	no	yes
9	59	aneuploid	d	C	rectum/left	poor	no	no
11	73	aneuploid	d	B	trs.col./right	moderate	no	no
14	46	aneuploid	n*	C	rectum/left	moderate	no	no
19	49	aneuploid	d	C	rectum/left	moderate	no	yes
22	81	aneuploid	d	C	asc.col./right	moderate	no	yes
CIN index < 18								
7	89	aneuploid	d	A	des.col.left	good	no	no
9′	59	diploid	d	C	rectum/left	poor	no	no
10	57	diploid	n	A	rectum/left	moderate	no	no
13	77	diploid	n	C	rectum/left	moderate	no	no
15	70	aneuploid	n*	B	rectum/left	moderate	no	no
16	57	diploid	n*	C	rectum/left	moderate	no	no
(b) p53 status, DNA content and clinicopathological data for RER+ colorectal cancers								
CIN index > 18								
12	60	diploid	d	C	asc.col./right	poor	no	yes
18	77	aneuploid	d	B	asc.col./right	poor	no	yes
CINindex < 18								
3	64	diploid	n	A	asc.col/right	moderate	no	no
17	76	diploid	n*	C	caecum/right	poor	no	no
20	70	diploid	n	C	asc.col./right	poor	yes	yes

n, p53 defect not detected by any of the three methods used; n*, IHC and mutation analysis negative, but p53 defect can not be excluded due to 17p loss detected by CGH; d, p53 defective (IHC or mutation analysis positive); des.col., descending colon; trs.col., transverse colon; sigm.col., sigmoid colon; asc.col., ascending colon

We next searched for differences in the patterns of specific clonal chromosomal abnormalities present in low- and high-CIN index RER− cancers. Analysis of chromosomal gains and losses in high-CIN index RER− cancers revealed the most frequent changes to be 20q+, 18q−, 13q+, 8p−, 1p− and 8q+. All of these changes were found, although less commonly, in the group of colorectal cancers with low CIN index (Table 3). Particularly striking, however, was the low incidence of 13q duplication in RER− tumours with

low CIN index (detected in 1/6 tumours) compared with RER− cancers with a high CIN index (10/11 tumours).

Comparison of primary tumours and xenografts

Analysis of RER+ primary tumours and their corresponding xenografts confirmed that microsatellite instability is a dynamic process. Sampling at multiple sites revealed that, although many of the sites sampled showed altered alleles at two or more of the four microsatellite loci tested, the affected loci and the shifts observed were often different at different sites within the same tumour. Xenografts showed further changes still. Thus, in the xenografts from three tumours, 17 of 44 tested microsatellite loci acquired clonal alleles different from the primary tumour within a single passage *in vivo* (Table 4).

To assess the progress of chromosomal changes, a scoring system similar to that for primary tumours was applied. For this purpose, we employed only those xenografts successfully established from at least two separate sites from the primary tumour (since this was a prerequisite for assessing the heterogeneity score). All of these tumours were either RER+ or RER− with a high CIN index. CIN index increased between primary tumour and corresponding xenograft, particularly in RER− cancers (Figure 3), suggesting the presence of a potent mechanism of underlying chromosomal instability in those RER− cancers which were established as xenografts. Interestingly, we failed completely to establish xenografts from all six of the RER− tumours with low CIN index, despite implanting multiple samples from each.

Discussion

We have used a combination of microsatellite instability, DNA ploidy and comparative genomic hybridization patterns to classify human primary colorectal carcinomas in terms of genomic instability. A further indication of the character of this instability has been gained by comparison of multiple samples gathered from the same tumours, and assessment of certain tumours after several weeks' regrowth as subcutaneous xenografts in SCID mice. Three distinct phenotypes emerge. The first – the RER+ phenotype – has been documented many times, both in primary tumours and cell lines. We confirm here the strikingly

Table 3 Most frequent chromosomal changes in RER− colorectal cancers

	All RER− tumours (n=17)	*RER− tumours with CIN>18 (n=11)*	*RER− tumours with CIN<18 (n=6)*
20q+	88% (15)*	100% (11)	67% (4)
18q−	76% (13)	91% (10)	50% (3)
13q+	65% (11)	91% (10)	17% (1)
8p−	59% (10)	73% (8)	33% (2)
8q+	53% (9)	64% (7)	33% (2)
1p−	53% (9)	64% (7)	33% (2)

*Figures are affected tumours as a percentage of all tumours in that category or (in brackets) as absolute numbers

Table 5 Oligonucleotide primer sequences and PCR annealing temperatures for amplification of p53 exons 5–8

p53 exon	*Primer sequence (5′–3′)*	*Annealing temperature*
5	TTCCTCTTCCTACAGTAGTC CCCAGCTGCTCACCATCG	55°C
6	CCTCACTGATTGCTCTTAGG AGTTGCAAACCAGACCTCAG	58°C
7	TGTGTTATCTCCTAGGTTGG TGGCAAGTGGCTCCTGAC	58°C
8	TCCTATCCTGAGTAGTGGT TCCTGCTTGCTTACCTCG	58°C

Table 4 Instability at four microsatellite loci recorded at multiple sites in RER+ tumours and their corresponding xenografts

	Locus							
	D2S123		*D13S160*		*BAT-26*		*TGFβ RII*	
Sample	*p*	*x*	*p*	*x*	*p*	*x*	*p*	*x*
3a	+ +	na	−	na	+	na	+	na
3b	+	na	−	na	+	na	+	na
3c	+	na	−	na	+ +	na	+	na
3d	+	na	−	na	+	na	+	na
12a	+	+*	+	+ +	+	+ +*	+	+*
12b	+	+*	+ +	+ +*	+	+*	+	+*
12c	+	+*	+	+	+	+ +*	+	+*
12d	+	+*	+ + +	+ + + +*	+	+*	+	+*
17a	−	na	+	na	+	na	+	na
17b	−	na	+	na	+ +	na	+	na
17c	+	na	+	na	+	na	+	na
17d	+ +	na	+ +	na	+ + +	na	+	na
18a	−	+	−	+	+ +	+ + +*	+	+*
18b	−	+	+	+ +*	+	+*	+	+*
18c	+	+ +	−	+*	+ + + +	+ + + +*	+	+*
18d	−	+	−	+	+ + +	+ + + +*	+	+*
20a	−	+*	+	+	+	+*	+	+
20b	−	na	−	na	+	na	+	na
20c	−	+ +*	+	+*	+	+*	+	+
20d	−	+*	−	−	+	+*	+	+

−, indicates no shift in allele size from normal; +, indicates a shift in allele size from normal, + +, + + +, + + + + indicate further shifts in allele size from normal; na, not available; p, primary tumour; x, xenograft; *, indicates complete loss of normal allele, noted only in xenografts

frequent generation of newly mutant subclones within these tumours, as identified by clonal errors at microsatellite sites. Although classically near diploid, the RER+ tumours were shown by CGH to include several chromosome arm amplifications and deletions. The number of such events per genome was low, however, as was the extent to which such chromosome structural changes varied in time or between adjacent sites within the same tumour.

The second phenotype is characterized by high incidence of chromosome arm amplification or deletion, as detected by CGH. These tumours are invariably RER−, and the chromosome instability clearly reflects a continuing state rather than the result of a catastrophic event early in tumour history: plurality and divergence of clonal chromosomal changes are evident both between adjacent sites and during growth in time. Although the chromosomal abnormalities in this group favour particular sites (which we have documented in more detail elsewhere), no chromosome arm is free from abnormality in the group as a whole, and the studies with xenografts clearly show that chromosomes that were normal in the primary tumour can appear as amplified or deleted in clonal outgrowths sampled only a few weeks later. This group probably corresponds broadly to the high CIN subgroup described by Lengauer *et al.* (1997), on the basis of counts of a restricted set of chromosome-specific centromeres. The CIN score developed here, however, detects a wider range of structural chromosome abnormalities, since the CGH method takes account of all chromosomes, and detects amplifications and deletions within chromosome arms as well as nondisjunctive lesions involving whole chromosomes. Unsurprisingly, all the tumours assigned to this group on the basis of their aberrant CGH profiles were also DNA aneuploid as assessed by flow cytometry.

The third phenotype combines RER− status with a propensity for change in chromosome arms as low as that in most RER+ tumours. For several reasons it is most improbable that these tumours are merely misclassified with respect to their RER status. The interrogated microsatellite sites included some of the most labile currently known, all the tumours were left-sided (most were rectal) and none showed mucinous differentiation. Neither is it probable that these tumours merely represent temporally early versions of the high CIN, RER− group. Of the six tumours assigned to this group, four had penetrated the muscle layer, three had lymph node metastases and all exceeded 3 cm in diameter at the time of study. Although there were some exceptions, many of the tumours in this group were near-diploid in DNA content. Thus, we consider it likely that these tumours arise by a pathway different from both the aneuploid RER− and the RER+ groups. Although our studies have not delineated a mutational mechanism, the near-diploid CGH patterns could accommodate point mutation, gene conversion, deletions and amplifications below the limits of resolution of CGH, balanced translocations or uniparental disomy.

We suspect that the RER− low CIN phenotype may be under-represented amongst currently available colorectal cancer cell lines, as RER− near-diploid lines are rare (Eshleman *et al.*, 1998). A recent study of primary tumours, however, has also identified a substantial subset of RER− colorectal carcinomas with diploid DNA content and therefore probably low CIN phenotype (Yao *et al.*, 1999). It may be that these tumours either fail to adapt to growth *in vitro* or, in doing so, undergo obligatory further changes in chromosome structure. In this respect, we were interested to note that none of our RER− low CIN tumours adapted to growth as xenografts, in contrast to RER− high CIN tumours and RER+ tumours studied around the same time.

Further work with large numbers of cases will be required to establish whether RER− low CIN tumours share a particular clinicopathological identity. It appears probable, however, that each of the three phenotypes described here arises because of deficiency in different checkpoint mechanisms. For RER+ tumours, the critical defect (failed recognition of DNA nucleotide mismatches) is known, but the corresponding defect or defects for the RER− tumour groups are still largely unknown. Information on this topic will be important, as it may be predictive of the efficacy of various therapeutic measures.

Materials and methods

Tissue samples

Fresh tissue samples were collected from consecutive sporadic colorectal carcinomas removed at operation between April and November 1997. Blocks of fresh tissue, approximately $10 \times 5 \times 5$ mm, were collected from two to four different sites, depending on the size of the tumour, from each colorectal cancer and one from normal mucosa at a point distant from the lesion. All tumours were from separate individuals except two (9 and 9′) which occurred synchronously. Each block of tissue was subsequently divided into three separate pieces: the first for DNA extraction and flow cytometry, the second for periodate-lysine-paraformaldehyde-dichromate (PLPD) fixation, and the third for xenografting (normal tissue was not xenografted). Sections from PLPD-fixed paraffin blocks were used for immunohistochemical detection of stabilized p53 protein and haematoxylin and eosin (H+E) staining for histological assessment. DNA was extracted from frozen tissue according to the method of Goelz *et al.* (1985).

Establishment of colorectal cancer xenografts in SCID mice

Xenografts were established from dorsal implants of freshly obtained tissue fragments, as previously described (McQueen *et al.*, 1991) but using severe combined immuno-deficient (SCID) mice as recipients. Tumours were allowed to grow until an externally visible diameter of about 1 cm was reached, and were then passaged to new hosts. At the time of passage, the mice were killed and the tumour tissue divided into pieces for DNA extraction, flow cytometry and PLPD fixation. H+E staining and immunohistochemical detection of stabilized p53 protein was carried out on fixed tissue. Implantation was attempted from each of the cohort of 74 tumour samples, of which 26 were successfully established as xenografts. Time to first passage varied between 3 and 20 weeks.

Flow cytometry

Frozen tissue was prepared for flow cytometry according to the method of Vindelov *et al.* (1983). Flow cytometry was performed on an EPICS-XL flow cytometer (Coulter Electronics Ltd, Luton, UK) at an excitation wavelength of 488 nm. At least 5000 nuclei were analysed in each sample,

and tumour DNA content estimated by comparison with identical analyses of normal tissue. Tumour samples were scored as 'DNA diploid' or 'DNA aneuploid' using previously described criteria (Carder *et al.*, 1993).

Immunohistochemical detection of stabilized p53 protein

Immunohistochemistry was carried out on 3 μm paraffin sections using the DO-7 antibody (Dako Ltd, UK) and an avidin-biotinylated horseradish peroxidase complex detection system (ABComplex/HRP, Dako Ltd, UK), with 3,3′-diaminobenzidine (DAB) as substrate, as previously described (Purdie *et al.*, 1991). Tumours were classified as p53 defective if more than 10% nuclei showed intense positive staining.

Mutation analysis of the p53 gene

Mutation analysis of the *p53* gene was performed on 15 tumours with faint, sparse or negative immunohistochemical staining to exclude the possibility of mutation not detectable by IHC. Previous studies have indicated that mutation in the *p53* gene, if present, occurs relatively early in tumour progression and therefore can be readily detected in any part of the tumour (Carder *et al.*, 1995). For this reason, a single sample was taken as representative of each tumour. Exons 5–8, in which 90% of all mutations are located (Levine *et al.*, 1991), were amplified using the primers listed in Table 5. Reactions were carried out in 50 μl volumes consisting of 200 ng genomic DNA, 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1 × PCR buffer solution (Life Technologies Ltd, UK) and 1.25 U of thermostable DNA polymerase (Life Technologies Ltd, UK) with the addition of 50 ng γ^{33}PdATP-labelled primer to each reaction in the final cycles.

SSCP with autoradiographic detection were undertaken as previously described (Carder *et al.*, 1995). Autoradiographs were assessed visually for shifts in electrophoretic mobility of amplified sequences compared to DNA from normal tissue of the same patient.

Analysis of microsatellite instability

Two dinucleotide repeat sequences, D2S123 and D13S160 (Gyapay *et al.*, 1994) and two poly(A) tracts, BAT-26 (Hoang *et al.*, 1997) and the $(A)_{10}$ repeat in exon 3 of TGFβ RII (using primers CCTCGCTTCCAATGAATCTC and TTGGCACAGATCTCAGGTCC), were analysed for evidence of microsatellite instability. All four loci were examined in each sampled site of 22 primary tumours and in all xenografts. Reactions were carried out as described above, with the addition of 10% DMSO, for all loci except BAT-26, where a final concentration of 100 μM of each dNTP with 450 ng genomic DNA template was employed, and TGFβ RII, where magnesium ion concentration was 4 mM. D2S123, D13S160 and poly(A) BAT-26 PCR products were heat-denatured and run on denaturing polyacrylamide gels to detect shifts in electrophoretic mobility. The $(A)_{10}$ sequence in exon 3 of TGF-β RII was assessed using SSCP as described above. Autoradiographs were assessed visually for the presence of shifts in electrophoretic mobility, comparing sequences from tumour samples and the corresponding normal DNA. Tumours were classified as RER+ if they displayed band shifts at two or more loci.

Analysis of imbalanced chromosomal abnormalities using comparative genomic hybridization

CGH was carried out using a method modified from Kallioniemi *et al.* (1992). Briefly, DNA was labelled by nick translation with biotin (tumour DNA) and digoxigenin (normal DNA), 500 ng of each prehybridized with 15 μg of human Cot-1 DNA, denatured and hybridized to a denatured normal male metaphase preparation for 2–3 days. Detection was carried out using avidin-FITC and anti-digoxigenin-rhodamine and slides were additionally stained with 4,6-diamidino-2-phenylindole (DAPI) to allow chromosome identification. Hybridizations were analysed using the Quantitative Image Processing System (QUIPS) software (Vysis Ltd, Richmond, Surrey, UK) coupled to a Zeiss Axioskop 20 fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) equipped with a SenSys CCD camera (Photometrics, Tucson, AZ, USA) and a triple bandpass filter set for detection of rhodamine, fluorescein and DAPI. At least five metaphase spreads were analysed from each slide. Because sex chromosomes might be under different selection pressure in tumours derived from males and females they were excluded from analysis. The ratios of 1.125 and 0.875 were used for scoring chromosomal gains and losses, ratios at which copy number changes could easily be visualized by eye. The same ratios were used for both primary tumours and xenografts, which could result in scoring of fewer chromosomal changes in the primary tumours due to contamination with normal stromal DNA. However, direct comparison of RER− and RER+ cancers could be made since the same error applied to both groups.

Acknowledgments

We are grateful to Harris Morrison of the Human Genetics Unit, MRC, Edinburgh for help and advice with CGH and to Joan Flanigan and Jennifer Doig for expert technical assistance. This study was funded by a Programme Grant from the Cancer Research Campaign [CRC] (Ref. no. 18201), the Medical Research Council and the Dr James and Bozena Bain fund.

References

Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B and de la Chapelle A. (1993). *Science*, **260**, 812–816.

Aaltonen LA, Peltomaki P, Mecklin JP, Jarvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M, Sistonen P, Hamilton SR, Kinzler KW, Vogelstein B and de la Chapelle A. (1994). *Cancer Res.*, **54**, 1645–1648.

Akiyama Y, Sato H, Yamada T, Nagasaki H, Tsuchiya A, Abe R and Yuasa Y. (1997). *Cancer Res.*, **57**, 3920–3923.

Borresen AL, Lothe RA, Meling GI, Lystad S, Morrison P, Lipford J, Kane MF, Rognum TO and Kolodner RD. (1995). *Hum. Mol. Gen.*, **4**, 2065–2072.

Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Erabino C, Lipford J, Lindblom A, Tannergard P, Bollag RJ, Godwin AR, Ward DC, Nordenskjold M, Fishel R, Kolodner R and Liskay RM. (1994). *Nature*, **368**, 258–261.

Bubb VJ, Curtis LJ, Cunningham C, Dunlop MG, Carothers AD, Morris RG, White S, Bird CC and Wyllie AH. (1996). *Oncogene*, **12**, 2641–2649.

Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JKV, Markowitz SD, Kinzler KW and Vogelstein B. (1998). *Nature*, **392**, 300–303.

Carder PJ, Cripps KJ, Morris R, Collins S, White S, Bird CC and Wyllie AH. (1995). *Br. J. Cancer*, **71**, 215–218.

Carder P, Wyllie AH, Purdie CA, Morris RG, White S, Piris J and Bird CC. (1993). *Oncogene*, **8**, 1397–1401.

Eshleman JR, Casey G, Kochera ME, Sedwick WD, Swinler SE, Veigl ML, Willson JKV, Schwartz S and Markowitz SD. (1998). *Oncogene*, **17**, 719–725.

Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Willson JKV, Veigl ML, Sedwick WD and Markowitz SD. (1995). *Oncogene*, **10**, 33–37.

Eshleman JR and Markowitz SD. (1996). *Hum. Mol. Gen.*, **5**, 1489–1494.

Eshleman JR, Markowitz SD, Donover PS, Lang EZ, Lutterbaugh JD, Li GM, Longeley M, Modrich P, Veigl ML and Sedwick WD. (1996). *Oncogene*, **12**, 1425–1432.

Goelz SE, Hamilton SR and Vogelstein B. (1985). *Biochem. Biophys. Res. Commun.*, **130**, 118–126.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, Bernardi G, Lathrop M and Weissenbach J. (1994). *Nature Genet.*, **7**, 246–339.

Hartwell L. (1992). *Cell*, **71**, 543–546.

Hoang JM, Cottu PH, Thuille B, Salmon RJ, Thomas G and Hamelin R. (1997). *Cancer Res.*, **57**, 300–303.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D and Perucho M. (1993). *Nature*, **363**, 558–561.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F and Pinkel D. (1992). *Science*, **258**, 818–821.

Kim HG, Jen J, Vogelstein B and Hamilton SR. (1994). *Am. J. Pathol.*, **145**, 148–156.

Konishi M, Kikuchi-Yanoshita R, Tanaka K, Muraoka M, Onda A, Okumura Y, Kishi N, Iwama T, Mori T, Koike M, Ushio K, Chiba M, Nomizu S, Konishi F, Utsunomiya J and Miyaki M. (1996). *Gastroenterology*, **111**, 307–317.

Lengauer C, Kinzler KW and Vogelstein B. (1997). *Nature*, **386**, 623–627.

Levine AJ, Momand J and Finlay CA. (1991). *Nature*, **351**, 453–456.

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomaki P, de la Chapelle A, Hamilton SR, Vogelstein B and Kinzler KW. (1996). *Nature Med.*, **2**, 169–174.

Loeb LA. (1991). *Cancer Res.*, **51**, 3075–3079.

Lothe RA, Peltomaki P, Meling GI, Aaltonen LA, Nystrom-Lahti M, Pylkkanen L, Heimdal K, Andersen TI, Moller P, Rognum TO, Fossa SD, Haldorsen T, Langmark F, Brogger A, de la Chapelle A and Borresen AL. (1993). *Cancer Res.*, **53**, 5849–5852.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun LZ, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M and Willson JKV. (1995). *Science*, **268**, 1336–1338.

McQueen HA, Wyllie AH, Piris J, Foster E and Bird CC. (1991). *Br. J. Cancer*, **63**, 94–96.

Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Mutaoka M, Yasuno M, Igari T, Koike M, Chiba M and Mori T. (1997). *Nature Genet.*, **17**, 271–272.

Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Dunlop MG, Hamilton SR, Petersen GM, de la Chapelle A, Vogelstein B and Kinzler KW. (1994). *Nature*, **371**, 75–80.

Nystrom-Lahti M, Parsons R, Sistonen P, Pylkkanen L, Aaltonen LA, Leach FS, Hamilton SR, Watson P, Bronson E, Cavalieri J, Lynch J, Lanspa S, Smyrk T, Lynch P, Drouhard T, Kinzler KW, Vogelstein B, Lynch HT, de la Chapelle À and Peltomaki P. (1994). *Am. J. Hum. Genet.*, **55**, 659–665.

Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Hamilton SR, Petersen GM, Watson P, Lynch HT, Peltomaki P, Mecklin JP, de la Chapelle A, Kinzler KW and Vogelstein B. (1994). *Science*, **263**, 1625–1629.

Peltomaki P, Lothe RA, Aaltonen LA, Pylkkanen L, Nystrom-Lahti M, Seruca R, David L, Holm R, Ryberg D, Haugen A, Brogger A, Borresen AL and de la Chapelle A. (1993). *Cancer Res.*, **53**, 5853–5855.

Purdie CA, O'Grady J, Piris J, Wyllie AH and Bird CC. (1991). *Am. J. Pathol.*, **138**, 807–813.

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. (1997). *Science*, **275**, 967–969.

Senba S, Konishi F, Okamoto T, Kashiwagi H, Kanazawa K, Miyaki M, Konishi M and Tsukamoto T. (1998). *Cancer*, **82**, 279–285.

Thibodeau SN, Bren G and Schaid D. (1993). *Science*, **260**, 816–819.

Thibodeau SN, French AJ, Cunningham JM, Tester D, Burgart LJ, Roche PC, McDonnell SK, Schaid DJ, Vockley CW, Michels VV, Farr Jr GH and O'Connell MJ. (1998). *Cancer Res.*, **58**, 1713–1718.

Togo G, Toda N, Kanai F, Kato N, Shiratori Y, Kishi K, Imazeki F, Makuuchi M and Omata M. (1996). *Cancer Res.*, **56**, 5620–5623.

Vindelov LL, Christensen IJ and Nissen NI. (1983). *Cytometry*, **3**, 323–327.

Wijnen J, Vasen H, Khan PM, Menko FH, van der Klift H, van Leeuwen C, van den Broek M, van Leeuwen-Cornelisse I, Nagengast F, Meijers-Heijboer A, Lindhout D, Griffioen G, Cats A, Kleibeuker J, Varesco L, Bertario L, Bisgaard ML, Mohr J and Fodde R. (1995). *Am. J. Hum. Genet.*, **56**, 1060–1066.

Wu C, Akiyama Y, Imai K, Miyake S, Nagasaki H, Oto M, Okabe S, Iwama T, Mitamura K, Masumitsu H, Nomizu T, Baba S, Maruyama K and Yuasa Y. (1994). *Oncogene*, **9**, 991–994.

Yao J, Eu KW, Seow-Choen F, Vijayan V and Cheah PY. (1999). *Int. J. Cancer*, **80**, 667–670.

Journal of Pathology
J Pathol 2000; **192**: 440–445.
DOI: 10.1002/1096-9896(2000)9999:9999<::AID-PATH761>3.0.CO;2-X

Original Paper

Specific patterns of chromosomal abnormalities are associated with RER status in sporadic colorectal cancer

Lucy J. Curtis*, Izabela B. Georgiades†, Susan White, Colin C. Bird, David J. Harrison and Andrew H. Wyllie‡
Sir Alastair Currie CRC Laboratories, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK

*Correspondence to: Dr L. J. Curtis, Sir Alastair Currie CRC Laboratories, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK.
E-mail: ljcurtis32@hotmail.com

†Current address: Department of Histopathology, The General Infirmary at Leeds, Great George Street, Leeds, LS1 3XE, UK.
‡Current address: Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK.

Received: 12 April 2000
Accepted: 26 June 2000
Published online: 4 October 2000

Abstract

Current opinion of the genetic events driving colorectal tumourigenesis focuses on genomic instability. At least two apparently independent mechanisms are recognized, microsatellite instability and chromosomal instability. The genetic defects underlying each type of instability are only partially understood and controversy remains as to the role of p53 in the generation of chromosomal defects in colorectal cancer. This study sought to clarify the relationships between chromosomal abnormalities and defects of both p53 and mismatch repair. Extensive chromosomal analysis was undertaken, using flow cytometry and comparative genomic hybridization, of a series of sporadic colorectal cancers which had been grown to early passage as subcutaneous xenografts in SCID mice. Overall levels of chromosomal defects were observed to be low in RER+ cancers compared with RER− and distinctive patterns of chromosomal anomalies were found to be associated with both the RER+ and RER− phenotype. No particular level or pattern of chromosomal anomalies appeared to be associated with p53 status, supporting recent observations that abnormal p53 function is not sufficient to cause chromosomal anomalies in colorectal tumours. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: **colorectal cancer; microsatellite instability; RER+; chromosome; ploidy; p53; CGH**

Introduction

Genomic instability appears to be fundamental to the development of malignancy. In sporadic colorectal cancer, two major mechanisms of genomic destabilization have been repeatedly demonstrated, involving either multiple chromosomal abnormalities or instability of microsatellite sequences (the RER+ phenotype) and other mechanisms may also exist [1]. Chromosomal instability, in the form of both non-disjunction and breakage events, has been demonstrated convincingly in cell lines derived from colorectal cancer [2,3] and it is likely that the clonal chromosomal abnormalities which are abundant in colorectal cancer [4–6] arise as a result of underlying defects of chromosome stability. An inverse correlation has been demonstrated in colorectal cancer cells *in vitro* between the RER+ phenotype and chromosomal instability [2,3] and evidence from primary sporadic colorectal cancers reflects this finding; RER+ tumours seldom show evidence for major abnormalities in chromosome structure or number [7–10] and if karyotypic abnormalities are present, they tend towards whole chromosomal duplications rather than structural anomalies [9]. It is believed that either type of instability is capable of driving tumour progression, although the two are not mutually exclusive.

The genetic basis of the RER+ phenotype has been studied extensively in patients with hereditary non-polyposis colorectal cancer (HNPCC), in whom the vast majority of cancers display this phenotype [7]. Germ-line defects of genes involved in the mismatch repair pathway account for almost all cases. A much lower proportion (around 15%) of sporadic colorectal cancers display the RER+ phenotype [11–14], of which most lack expression of a mismatch repair protein, hMLH1 [15]. The genetic basis for chromosomal instability is unclear. Certain cell lines displaying this phenotype have been shown to be defective in a kinetochore checkpoint function, potentially facilitating chromosomal non-disjunction, and a proportion of these cell lines harbour mutations in a gene encoding one component of this checkpoint, *hBUB1* [16]. Abnormalities of p53, present in at least 70% of sporadic colorectal cancers [17,18], could also be involved in the acquisition of chromosomal defects, since there is compelling evidence from many sources linking abnormalities of the p53 protein with both numerical and structural chromosome abnormalities [19–22]. Furthermore, disruption of p53 function has been shown to precede aneuploid clonal divergence in colorectal cancer [23,24]. A number of mechanisms by which p53 helps to maintain genomic stability have been postulated, including its involvement in a mitotic spindle checkpoint and in the p21-mediated G1 checkpoint, preventing the propagation of cells in which DNA is damaged. However, recent analyses of

colorectal cancer cell lines suggest that there is no direct correlation between abnormalities of p53 and chromosomal instability [2,3].

In this paper we seek to clarify the roles of defects of p53 and of the mismatch repair pathway in the generation of chromosomal abnormalities in primary tumour colorectal cancer. Recent studies of genomic instability have gathered much important data from the analysis of relatively small numbers of cell lines derived from colorectal cancers Whilst cells grown *in vitro* are a valuable resource, they may not be entirely representative of the spectrum of colorectal cancers, as they are exposed to selective pressures very different from those in tumours *in vivo*. We chose, therefore, to analyse chromosomal defects in a series of samples of authentic tumour tissue. We utilized tumours grown to early passage as xenografts so as to eliminate human stromal contamination from tumour material. Tumours grown in this way show closely similar DNA ploidy to that of the primary tumour from which they are established and have the advantage that the neoplastic cells are not admixed with normal human cells [25,26]. Our results confirm that chromosomal abnormalities occur less often in RER+ cancers than in RER−, but are not obviously related to the status of p53. Furthermore, a pattern of chromosomal copy number changes has emerged in which certain chromosomal abnormalities are grossly overrepresented in RER− cancers, whilst others are overrepresented in RER+, indicating differences between RER− and RER+ cancers either in the mechanisms by which chromosome anomalies occur, or in the selection pressures for particular chromosomal abnormalities.

Materials and methods

Tissue samples

Tissue was harvested fresh from sporadic colorectal carcinomas removed at elective operation between 1988 and 1994 at Edinburgh Royal Infirmary. Some tissue was frozen at −70°C and the remainder fixed in periodate–lysine–paraformaldehyde–dichromate (PLPD) and embedded in paraffin wax. Thirty-six fresh primary sporadic colorectal carcinomas were established as subcutaneous xenografts by dorsal implantation in severe combined immunodeficient (SCID) mice as previously described [26]. DNA was extracted from frozen tissue according to the method of Goelz *et al.* [27] from xenografts harvested at early passage, pass 1 or 2, except in five cases where passes 3 (three cases), 4, and 5 were used.

Analysis of chromosomal abnormalities

Comparative genomic hybridization (CGH) [28,29] was used to evaluate abnormalities in copy number of chromosome arms and flow cytometry was employed to determine DNA ploidy. CGH was effected by a modified version of the method described by Kallioniemi *et al.* [28]. Briefly, a normal male metaphase spread was treated with 10 μg/ml RNase A for 1 h, 100 ng/ml proteinase K for 2.5 min, and 70% formamide at 70°C for 3 min, and dehydrated through 70%, 90%, and 100% ethanol. 500 ng of tumour DNA was labelled with biotin by nick translation and counter-hybridized against 500 ng of digoxigenin-labelled normal DNA and 15 μg of Cot-1 DNA for 2 days. A rhodamine-conjugated anti-digoxigenin antibody and fluorescein-conjugated avidin were used to visualize hybridized normal and tumour DNA. Hybridizations were analysed using the image analysis software MacProbe (Perceptive Scientific Instruments Ltd., Chester, UK) or QUIPS [Vysis (UK) Ltd., Richmond, Surrey, UK]. Between five and ten metaphase spreads were analysed for each tumour and green/red ratio cut-off points of 1.125 and 0.875 were chosen for scoring of chromosome copy number changes, the level at which copy number changes could easily be visualized by eye. Sex chromosomes were omitted from statistical analysis because of the bias against X-chromosome loss in males.

Kallioniemi *et al.* [30] suggest that some chromosomal regions, namely 1p32-pter, 16p, 19, and 22, can give aberrant results, probably because of their high guanine/cytosine content. In this study, normal versus normal controls, which were included in each experiment, usually demonstrated even hybridization of DNA to all chromosomes, although chromosome 19 occasionally gave aberrant results. Reversal of the usual red to green labelling of tumour and normal DNA demonstrated chromosome 19 to be the only region of inconsistency. Hence, although our results from this chromosome are included, they are interpreted cautiously.

Preparation of frozen tissue for flow cytometry was carried out according to the method of Vindelov *et al.* [31] and performed on an EPICS-XL flow cytometer (Coulter Electronics Ltd., Luton, UK) at an excitation wavelength of 488 nm.

Analysis of microsatellite instability

Microsatellite instability was assessed by analysis of at least four of the following loci: D2S123 [32], D3S1293 [32], D8S282 [32], D13S160 [32], BAT26 [33], and TGFβRII (using oligonucleotide primer sequences CCTCGCTTCCAATGAATCTC and TTGGCACA GATCTCAGGTCC). Analysis was carried out by PCR amplification, denaturing polyacrylamide gel electrophoresis, and silver staining as previously described [14]. Tumours were designated positive for RER+ when allele length changes were observed at two or more loci.

Analysis of p53 status

Immunohistochemical staining of p53 was carried out using two antibodies, pAb1801 (Oncogene Science, UK) and DO7 (Dako Ltd., UK). Immunohistochemistry (IHC) was performed on 3 μm PLPD/paraffin

sections. Tumours were deemed to be positive if more than 10% positive nuclei were scored; this excluded cases in which only occasional nuclei were positive. In order to clarify the status of p53 in tumours which scored negative by these criteria or in which numbers of IHC-positive nuclei were low, SSCP mutation analysis of exons 5–8 of the gene, the region in which more than 90% of mutations are found in colorectal cancer [34], was carried out as previously described [35]. Thus, p53 status was deemed abnormal on the basis of either positive IHC or direct identification of a mutation in exons 5–8, or both.

Oligonucleotide primer sequences for p53 exons 5–8 were (5′ to 3′): exon 5 forward TTCCTCTTCCTA CAGTAGTC, reverse CCCAGCTGCTCACCATCG; exon 6 forward CCTCACTGATTGCTCTTAGG, reverse AGTTGCAAACCAGACCTCAG; exon 7 forward TGTGTTATCTCCTAGGTTGG, reverse TGG CAAGTGGCTCCTGAC; and exon 8 forward TCC TATCCTGAGTAGTGGT, reverse TCCTGCTTGC TTACCTCG.

Results

Chromosomal abnormalities determined by CGH

Analysis of tumours by CGH revealed both numerical chromosomal abnormalities and those involving breakage events, though it was notable that many breakage events occurred in centromeric regions. In keeping with cytogenetic and other CGH studies [4,5], overall common chromosomal abnormalities were found to be gain of chromosome arms 7p, 7q, 8q, 13q, and 20q, and loss of chromosome arms 8p and 18q. Certain chromosomal events, namely deletion of chromosome arms 8p and 18q and duplication of chromosome arms 8q, 13q, and 20q, were notable in that they could usually still be observed when the CGH cut-off ratio was increased to the equivalent of loss or gain of one chromosome in 75% diploid cells, indicating their presence as the majority clonal population.

Chromosomal abnormalities in RER+ and RER− colorectal tumours

Nine tumours of the 36 in the series displayed the RER+ phenotype. The characteristics of the cancers in this series with widespread microsatellite instability conformed to the predicted phenotype, in that they were all derived from the proximal colon (compared with RER−, $p=0.0012$, two-tailed Fisher's exact test). The average age of these patients (69.2 years for RER+, 67.7 years for RER−) and the proportions of cancers of Dukes' stage A, B, and C/D were similar in RER+ and RER− tumours. Clinico-pathological and genetic characteristics of the tumours are shown in Table 1.

Although the number of chromosomal changes detected by CGH, scored as the sum of each chromosome arm which contained a gain or a loss, was higher in RER− cancers than in RER+, this did not reach statistical significance ($p=0.111$, Mann–Whitney test, mean values 14.52 and 9.44, respectively). When DNA ploidy changes, as detected by flow cytometry, were analysed, a similar picture emerged; RER− cancers were usually aneuploid (24/27 tumours) whereas most (7/9) RER+ cancers were near-diploid ($p<0.001$, two-tailed Fisher's exact test). However, some unequivocal RER+ tumours were aneuploid by flow cytometry and most showed some abnormality in chromosomes as judged by CGH. Similarly, whilst most RER− tumours were aneuploid, several showed a relatively low number of chromosome arm gains and losses within the range observed in RER+ tumours. Moreover, the pattern of chromosomal change observed in the two tumour groups also differed. Overall, duplication of chromosome 7 and arms 8q, 13q, and 20q, and deletion of chromosome arms 8p and 18q were frequently observed (Figure 1). However, loss of 8p, which was observed with striking frequency in RER− tumours (20/27, 74%), was not present in any of the nine RER+ tumours. RER+ tumours showed loss of 18q in only one of nine tumours (11%) compared with 22/27 (81%) RER− cancers. Only two of the 27 RER− cancers displayed neither 8p nor 18q deletion. Abnormalities of chromosome 19 occurred in 5/9 RER+ cancers (compared with only 3/27 RER−), but the uncertainty regarding interpretation of changes in this chromosome has already been referred to.

Chromosomal abnormalities in relation to defects of p53

Twenty-four tumours were positive for abnormalities of p53, assessed either by the detection of a mutation within p53 or more than 10% positive nuclei by immunohistochemistry. The percentage of immunohistochemically positive nuclei per positive tumour varied from less than 1% to more than 90%. This wide variation in levels of staining has been reported repeatedly. There was a consistent strong association between mutation of the gene and the presence of over 30% more of positive-staining nuclei [36,37]. This group included five tumours which showed negative or very low levels of nuclear staining by IHC, but harboured mutations within exons 5 or 6. Of tumours with defective p53, 13/24 were left-sided compared with 4/12 with apparently normal p53, but this apparent difference was not statistically significant ($p=0.409$, Yates' corrected χ^2 test). Nor were there significant differences in Dukes' stages when tumours with normal and abnormal p53 were compared (15/24 with defective p53 were stage C/D compared with 3/12 with normal p53, $p=0.077$, Yates' corrected χ^2 test). The average age of patients was similar in both the p53-normal and -abnormal tumour groups (67.17 and 68.54 years, respectively).

The numbers of chromosomal changes assessed by CGH were not significantly different in p53-abnormal and -normal tumours ($p=0.602$, Mann–Whitney test, mean values 13.46 and 12.75, respectively). The proportions of aneuploid and diploid tumours were also

Table 1. Clinico-pathological, molecular, and immunocytochemical data for colorectal cancer xenografts

Tumour	Age (years)	Side	Site	Dukes' stage	RER status	p53 status	17p loss	DNA content by flow cytometry	No. of dups CGH	No. of dels CGH	Total dups+ dels CGH
1	71	Right	Cae	C/D*	RER+	Defective	Yes	Diploid	2	2	4
2	82	Right	Asc	B	RER+	Defective	Yes	Aneuploid	3	8	11
3	81	Right	Cae	B	RER+	Defective	No	Diploid	3	1	4
4	60	Right	Asc	C/D*	RER+	Defective	No	Diploid	5	6	11
5	68	Right	Asc	B	RER+	Normal	Yes	Diploid	1	0	1
6	49	Right	Cae	B	RER+	Normal	Yes	Diploid	8	13	21
7	65	Right	Cae	B	RER+	Normal	No	Diploid	2	0	2
8	77	Right	Cae	B	RER+	Normal	No	Aneuploid	8	13	21
9	70	Right	Asc	C/D*	RER+	Normal	Yes	Diploid	5	4	9
10	77	Right	Asc	B	RER−	Defective	No	Aneuploid	6	1	7
11	83	Right	Cae	C/D*	RER−	Defective	No	Diploid	4	10	14
12	84	Right	Cae	C/D*	RER−	Defective	Yes	Aneuploid	4	7	11
13	85	Right	Asc	C/D*	RER−	Defective	Yes	Aneuploid	7	5	12
14	48	Right	Asc	C/D*	RER−	Defective	No	Aneuploid	5	5	10
15	81	Right	Asc	C/D*	RER−	Defective	No	Aneuploid	7	4	11
16	65	Left	Rec	C/D*	RER−	Defective	Yes	Aneuploid	14	6	20
17	66	Left	Rec	C/D*	RER−	Defective	Yes	Aneuploid	6	12	18
18	36	Left	Sig	C/D*	RER−	Defective	No	Aneuploid	6	3	9
19	40	Left	Sig	C/D*	RER−	Defective	Yes	Aneuploid	6	4	10
20	68	Left	Des	B	RER−	Defective	Yes	Diploid	5	15	20
21	78	Left	Sig	B	RER−	Defective	No	Aneuploid	5	7	12
22	86	Left	Sig	B	RER−	Defective	No	Aneuploid	11	10	21
23	56	Left	Sig	B	RER−	Defective	No	Aneuploid	3	6	9
24	61	Left	Sig	C/D*	RER−	Defective	Yes	Aneuploid	5	5	10
25	40	Left	Rec	C/D*	RER−	Defective	No	Aneuploid	8	2	10
26	95	Left	Sig	B	RER−	Defective	Yes	Aneuploid	6	12	18
27	72	Left	Rec	B	RER−	Defective	Yes	Aneuploid	11	9	20
28	49	Left	Rec	C/D*	RER−	Defective	No	Aneuploid	10	16	26
29	81	Right	Asc	C/D*	RER−	Defective	Yes	Aneuploid	7	18	25
30	39	Right	Cae	B	RER−	Normal	Yes	Diploid	1	5	6
31	71	Right	Asc	C/D*	RER−	Normal	Yes	Aneuploid	11	13	24
32	87	Left	NK	A	RER−	Normal	No	Aneuploid	0	1	1
33	68	Left	Sig	B	RER−	Normal	No	Aneuploid	4	1	5
34	76	Left	Sig	A	RER−	Normal	Yes	Aneuploid	9	5	14
35	55	Right	Asc	C/D*	RER−	Normal	No	Aneuploid	19	16	35
36	81	Left	Sig	B	RER−	Normal	No	Aneuploid	7	7	14

*Tumours were classified as Dukes' C/D because surgical reports permitting classification as Dukes' D were not always available.
Abbreviations: Cae–caecum; Asc–ascending colon; Des–descending colon; Sig–sigmoid colon; Rec–rectum; dups–duplications; dels–deletions.

similar when cancers with defective p53 were compared with normal ($p=0.247$, Fisher's exact test). Abnormalities of p53 appeared more common in RER − tumours than in RER +, occurring in 20/27 (74%) and 4/9 (44%), respectively, but this difference was not statistically significant ($p=0.126$, Fisher's exact test).

Because loss of heterozygosity of 17p might indicate abnormal p53 function, we repeated the above tests, this time adding to the group defined as 'abnormal for p53' all cancers in which loss of 17p was identified by CGH. Using these criteria, only six tumours were classified as normal for p53. The tumours in this small group did not differ significantly in terms of ploidy, location, Dukes' stage or chromosomal alterations at other sites from those in which p53 was defective.

Chromosomal abnormalities by CGH in relation to DNA ploidy

The number of chromosome arms exhibiting abnormalities by CGH was higher in tumours found to be aneuploid by flow cytometry than in those which were diploid, though this did not reach statistical significance ($p=0.536$, Mann–Whitney test, mean values 14.77 and 9.2, respectively). Diploid and aneuploid tumours showed similar patterns of chromosomal abnormalities (Table 2).

Table 2. Frequency of chromosomal abnormalities detected by CGH in diploid or aneuploid colorectal tumours scored on the basis of DNA flow cytometry

Chromosomal abnormality	No. (%) of tumours with abnormality	
	Aneuploid	Diploid
+7	19 (73)	3 (30)
−8p	18 (69)	2 (20)
8q+	15 (58)	5 (50)
13q+	19 (73)	3 (30)
18q−	20 (77)	3 (30)
20q+	19 (73)	2 (20)

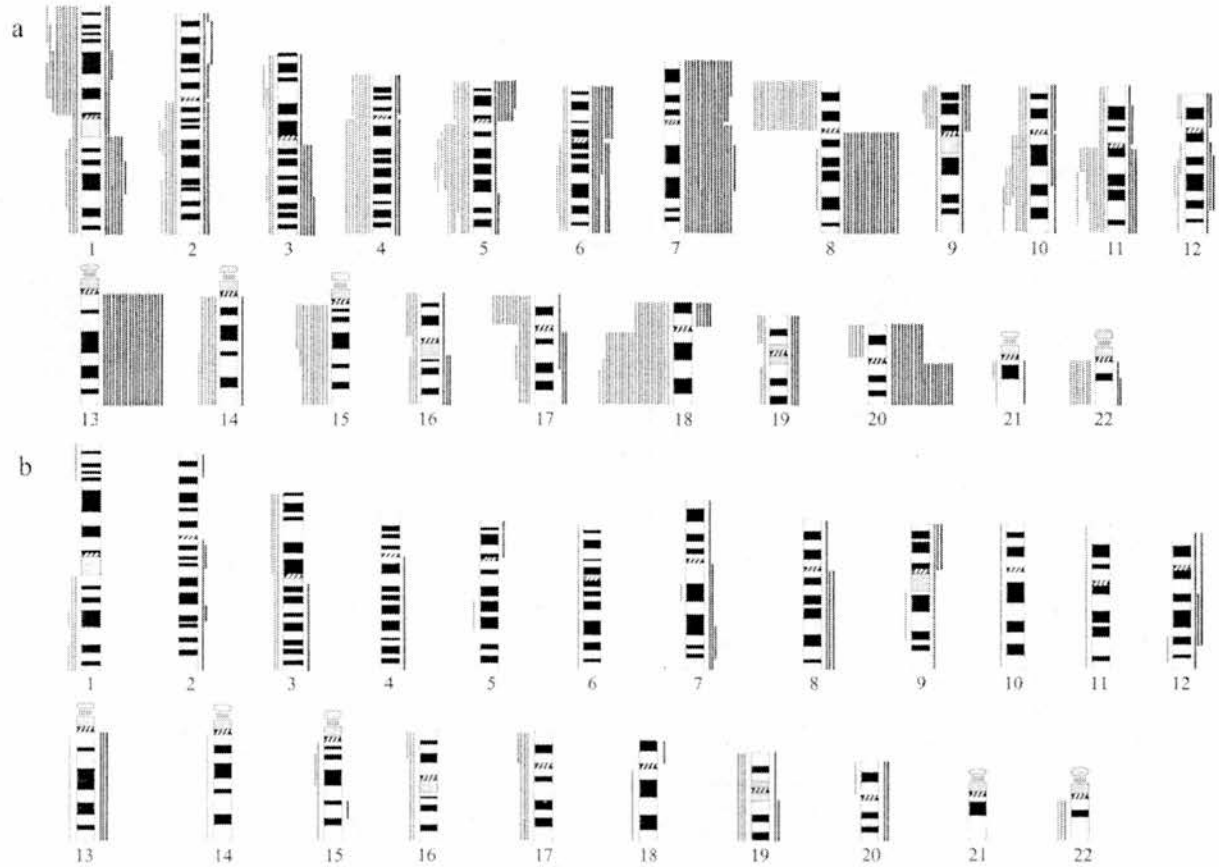


Figure 1. Chromosome duplications and deletions by CGH in early passage xenografts derived from sporadic colorectal cancers. Deletions are represented by grey bars to the left of each chromosome, duplications by black bars to the right. (a) Chromosomal changes in RER− cancers. The most frequent chromosomal abnormalities were deletion of chromosome arms 18q (81%) and 8p (74%), and duplication of all or part of chromosome 7 (70%) and chromosome arms 13q (70%), 20q (70%), and 8q (63%). (b) Chromosomal changes in RER+ cancers. Duplication of all or part of chromosome 7 occurred in 22% of RER+ tumours and duplication of 8q in 33%, 13q in 33%, and 20q in 22%. Deletion of chromosome arm 18q occurred in 11% of RER+ cancers and deletion of 8p was not observed

Discussion

Our data demonstrate appreciable numbers of chromosomal anomalies in RER+ cancers, as determined by CGH. These changes clearly differ from those in RER− cancers, in that they occur at a lower overall frequency (though this difference is not statistically significant), are generally present against a background of diploid DNA content (compared with the grossly aneuploid content of the majority of RER− tumours), and tend not to include the same pattern of frequent chromosomal abnormalities seen in RER− cancers, most notably loss of arms 8p and 18q. It is not apparent whether chromosomal abnormalities in RER+ colorectal cancers are caused by a generalized defect in chromosomal instability, though at least one form of chromosomal instability exists in conjunction with microsatellite instability in cell lines derived from colorectal cancer [2]. If this is the case, there is little clonal growth of cells which harbour the abnormalities prevalent in RER− tumours (involving chromosomes 7, 8, 13, 18, and 20). Selection pressure in favour of these anomalies may be diminished if the RER+ phenotype predominates in driving tumourigenesis, such that defects of mismatch repair cause mutations within critical oncogenes and tumour suppressor genes at an early stage.

Abnormality of a single gene product with multiple functions in chromosome stability regulation, such as p53, could allow chromosomal instability to arise. We found no association between abnormal chromosome content, assessed either by flow cytometry or by CGH, and defects of p53 in this series. This is in keeping with the data of de Angelis *et al.* [6] and supports data from other recent studies which suggest that although p53 may play some role in the propagation of chromosomal defects, other fundamental causative factors exist [1–3].

In summary, abnormal p53 status does not appear to differentiate any particular pattern of chromosomal change. Significant chromosomal changes occur in RER+ tumours, usually around a near-diploid total DNA content, but these do not harbour the very distinctive patterns of chromosomal changes observed in RER− tumours. These data suggest that the mechanisms by which chromosomal abnormalities arise or are selected for are dependent on RER status and as such provide further insight into the

mechanisms of genomic instability present in these tumours.

Acknowledgements

We are grateful to Harris Morrison of the Human Genetics Unit, MRC, Edinburgh for help and advice with CGH and to Joan Flanigan and Jennifer Doig for expert technical assistance. This study was funded by a programme grant from the Cancer Research Campaign (CRC; Ref. No. 18201), the Medical Research Council, and the Scottish Hospital Endowment Research Trust.

References

1. Georgiades IB, Curtis LJ, Morris RM, Bird CC, Wyllie AH. Heterogeneity studies identify a subset of sporadic colorectal cancers without evidence for chromosomal or microsatellite instability. *Oncogene* 1999; **18**: 7933–7940.
2. Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**: 623–627.
3. Eshleman JR, Casey G, Kochera ME, *et al.* Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of p53. *Oncogene* 1998; **17**: 719–725.
4. Muleris M, Salmon RJ, Dutrillaux B. Cytogenetics of colorectal adenocarcinomas. *Cancer Genet Cytogenet* 1990; **46**: 143–156.
5. Ried T, Knutzen R, Steinbeck R, *et al.* Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes Cancer* 1996; **15**: 234–245.
6. De Angelis PM, Clausen OPF, Schjølberg A, Stokke T. Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy., genotypes and phenotypes. *Br J Cancer* 1999; **80**: 526–535.
7. Aaltonen LA, Peltomaki P, Leach FS, *et al.* Clues to the pathogenesis of familial colorectal cancer. *Science* 1993; **260**: 812–816.
8. Lothe RA, Peltomaki P, Meling GI, *et al.* Genomic instability in colorectal cancer–relationship to clinicopathological variables and family history. *Cancer Res* 1993; **53**: 5849–5852.
9. Remvikos Y, Vogt N, Muleris M, Salmon RJ, Malfoy B, Dutrillaux B. DNA repeat instability is associated with colorectal cancers presenting minimal chromosome rearrangements. *Genes Chromosomes Cancer* 1995; **12**: 272–276.
10. Schlegel J, Stumm G, Scherthan H, *et al.* Comparative genomic *in situ* hybridization of colon carcinomas with replication error. *Cancer Res* 1995; **55**: 6002–6005.
11. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; **363**: 558–561.
12. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993; **260**: 816–819.
13. Kim HG, Jen J, Vogelstein B, Hamilton SR. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *Am J Pathol* 1994; **145**: 148–156.
14. Bubb VJ, Curtis LJ, Cunningham C, *et al.* Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene* 1996; **12**: 2641–2649.
15. Thibodeau SN, French AJ, Cunningham JM, *et al.* Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of *hMLH1*. *Cancer Res* 1998; **58**: 1713–1718.
16. Cahill DP, Lengauer C, Yu J, *et al.* Mutations of mitotic checkpoint genes in human cancers. *Nature* 1998; **392**: 300–303.
17. Baker SJ, Preisinger AC, Jessup JM, *et al.* p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* 1990; **50**: 7717–7722.
18. Cunningham J, Lust JA, Schaid DJ, *et al.* Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Res* 1992; **52**: 1974–1980.
19. Bischoff FZ, Yim SO, Pathak S, *et al.* Spontaneous abnormalities in normal fibroblasts from patients with Li–Fraumeni cancer syndrome – aneuploidy and immortalization. *Cancer Res* 1990; **50**: 7979–7984.
20. Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* 1992; **70**: 923–935.
21. Yin YX, Tainsky MA, Bischoff FZ, Strong LC, Wahl GM. Wild-type p53 restores cell-cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* 1992; **70**: 937–948.
22. Agapova LS, Ilyinskaya GV, Turovets NA, Ivanov AV, Chumakov PM, Kopnin BP. Chromosome changes caused by alterations of p53 expression. *Mutat Res* 1996; **354**: 129–138.
23. Carder P, Wyllie AH, Purdie CA, *et al.* Stabilized p53 facilitates aneuploid clonal divergence in colorectal cancer. *Oncogene* 1993; **8**: 1397–1401.
24. Carder PJ, Cripps KJ, Morris R, *et al.* Mutation of the p53 gene precedes aneuploid clonal divergence in colorectal carcinoma. *Br J Cancer* 1995; **71**: 215–218.
25. LeFrancois D, Olschwang S, Delattre O, *et al.* Preservation of chromosome and DNA characteristics of human colorectal adenocarcinomas after passage in nude mice. *Int J Cancer* 1989; **44**: 871–878.
26. McQueen HA, Wyllie AH, Piris J, Foster E, Bird CC. Stability of critical genetic lesions in human colorectal carcinoma xenografts. *Br J Cancer* 1991; **63**: 94–96.
27. Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem Biophys Res Commun* 1985; **130**: 118–126.
28. Kallioniemi A, Kallioniemi OP, Sudar D, *et al.* Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992; **258**: 818–821.
29. Du Manoir S, Speicher MR, Joos S, *et al.* Detection of complete and partial chromosome gains and losses by comparative genomic *in situ* hybridization. *Hum Genet* 1993; **90**: 590–610.
30. Kallioniemi OP, Kallioniemi A, Piper J, *et al.* Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosomes Cancer* 1994; **10**: 231–243.
31. Vindelov LL, Christensen IJ, Nissen NI. A detergent–trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1983; **3**: 323–327.
32. Gyapay G, Morissette J, Vignal A, *et al.* The 1993–94 Genethon human genetic linkage map. *Nature Genet* 1994; **7**: 246–339.
33. Hoang JM, Cottu PH, Thuille B, Salmon RJ, Thomas G, Hamelin R. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Res* 1997; **57**: 300–303.
34. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature* 1991; **351**: 453–456.
35. Cripps KJ, Purdie CA, Carder PJ, *et al.* A study of stabilization of p53 protein versus point mutation in colorectal carcinoma. *Oncogene* 1994; **9**: 2739–2743.
36. Baas IO, Mulder JWR, Offerhaus GJA, Vogelstein B, Hamilton SR. Evaluation of 6 antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994; **172**: 5–12.
37. Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D, Barnes DM. Analysis of p53 expression in human tumors – an antibody raised against human p53 expressed in *Escherichia coli*. *J Cell Sci* 1992; **101**: 183.